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(54) Title: AB_5 TOXIN B SUBUNIT MUTANTS WITH ALTERED CHEMICAL CONJUGATION CHARACTERISTICS

(57) Abstract: A recombinant AB_5 B subunit protein including at least one mutation, wherein the mutation alters the number of amino acid residues available for chemical modification as compared to a wild type AB_5 B subunit protein, and wherein said recombinant protein retains an effective target ligand bind affinity. For example, specifically designed mutations are produced in the cholera Toxin B subunit (CTB) such that it can still bind with high affinity to its receptor, Gm-1, but can be specifically covalently linked at lysines or cysteines to an immunogen or vaccine. The vaccine produced from this coupling is a mucosal vaccine which has high immunogenicity due to the interaction with the CTB. The vaccine can be produced inexpensively and easily. Alternatively, a technique is disclosed for treating CTB such that non-covalent coupling to a vaccine or immunogen can occur.

AB_5 TOXIN B SUBUNIT MUTANTS WITH ALTERED CHEMICAL CONJUGATION CHARACTERISTICS

FIELD OF THE INVENTION

This invention relates to methods of creating mutants in AB_5 toxin B subunit proteins, such as cholera toxin B subunit (CTB), with altered chemical conjugation characteristics. Compositions comprising the mutant AB_5 toxin B subunit proteins are also discussed. Further, use of these compositions as vaccines and bioactive molecule delivery agents is also discussed.

BACKGROUND OF THE INVENTION

Mucosal Immunity

Most licensed vaccines are delivered parenterally, a route of immunization that, in general, is extremely effective at inducing systemic protection against invasive disease. However, as many pathogens replicate or invade at mucosal surfaces, the efficacy of vaccines against these organisms may be improved if both systemic and mucosal immune responses were induced. Vaccines may have to be administered at a mucosal site, for example by oral or nasal delivery, in order to induce mucosal immune responses. Indeed, this may be a prerequisite for the production of vaccines against certain pathogens for which no vaccine is currently available (e.g., respiratory syncytial virus and even possibly HIV). An additional potential advantage of mucosal vaccines is that they would be easier to deliver, an extremely important consideration in mass immunization programs.

Unfortunately, delivery of many antigens via mucosal routes is often characterized by low immune responsiveness, due in part to inefficient uptake of antigen. As a result, various strategies for effective delivery of antigens by mucosal routes have been investigated. Antigens that are successfully delivered across the barrier of epithelial cells lining mucosal tracts stimulate underlying inductive sites of the mucosa-associated lymphoid tissue (MALT). Antigen-specific lymphocytes that are sensitized in the MALT migrate through the circulatory system to populate distant mucosal sites, thus mucosal immunization may provide both local and systemic protection.

Because many microorganisms invade their hosts via mucosal membrane surfaces, the development of a protective mucosal immune response can provide a preemptory line of defense against a variety of infectious diseases. Successful oral vaccination must induce the production of mucosal antibodies and/or cellular immunity. However, oral administration of many purified antigens fails to result in any substantial immune response.

Use of cholera toxin in mucosal immunity

Cholera toxin (CT) has long been considered responsible for inducing an effective, long-lasting protective mucosal immune response to subsequent cholera infection and disease. It was soon discovered that an enhanced mucosal immune response to orally administered antigens can be

achieved when the antigens are mixed with CT or conjugated to cholera toxin B protein (CTB). The ability of CT and CTB to act as mucosal adjuvants may stem from the adhesive and epithelial cell membrane-permeabilizing effects of these proteins; however, it appears that active toxin is more effective than the B subunit in exerting this immunostimulatory activity. It is therefore important to determine the events associated with CTB-membrane interaction and to identify mechanisms related to the potential translocational pathways of CTA1.

Cholera Toxin

The overt symptoms of cholera are well-known and the cellular events responsible for toxicity of the *Vibrio cholerae* enterotoxin (CT) have been thoroughly described. In contrast, molecular details of how the different subunits of the hexameric AB_5 complex of CT act together to initiate cellular toxicity remain poorly understood. What is known is that CT (M_r = 85 620) binds to gangliosides on the apical surface of intestinal epithelial cells via its homopentameric B subunit (CTB). The unusually strong avidity of CTB for gangliosides, particularly for GM-1, is most likely due to the cooperative affinities of each of the monomeric subunits in their pentameric form. Upon binding, CTB forms a planar ring structure on the membrane surface which possesses a highly polar central pore that interacts intimately with the A subunit of CT. The A subunit is composed of a toxic CTA1 subunit linked to the CTA2 subunit by a disulfide bond. Liberation of CTA1 by reduction of this disulfide linkage occurs during transcytosis through the cell to its basolateral membrane. This leads to permanent activation of adenylate cyclase and ribosylation of the GTP-binding regulatory protein $G_{S\alpha}$. These events result in the activation of sodium pumps via cAMP-dependent protein kinases and subsequent expulsion of sodium and water into the intestinal lumen. While CTB is clearly responsible for toxin binding to ganglioside GM-1 receptors, an essential step in cholera toxicity, the mechanism for transport of CTA1 into the cytoplasmic compartment of target cells, remains unclear.

Studies of CT-membrane interactions have shown that CTB binding to GM-1 incorporated into lipid bilayers induces the formation of membrane channels. These findings suggest that CTA1 is translocated into the target cell cytoplasm by passing through a protein-lined pore that traverses the membrane. However, on the basis of investigations using membrane-imbedded photoactivatable probes, as well as low-resolution structural studies, it does not appear that CTB enters the membrane following its association with GM-1 receptors. These studies could indicate that CT binds with CTA1 oriented toward the cell surface, thereby forcing CTA1 into and through the membrane upon CTB-GM-1 interaction; however, such a mechanism is not consistent with the crystal structures of CT, CTB, and the closely related heat-labile enterotoxin of *Escherichia coli* (LT). High-resolution structural analyses show that if the B pentamer of the toxin were bound with the A1 polypeptide oriented toward the membrane, a significant portion of the B subunit would

also have to enter the membrane. Moreover, recent evidence has demonstrated that functional CT binds with CTA1 facing away from the cell surface. These observations and the fact that CT does not appear to undergo significant structural changes upon receptor association have prompted many questions concerning the mechanism by which CTA1 moves from a site that is some distance from the cell surface to a position within the target cell cytoplasm.

Evidence is now accumulating that supports a model in which endocytosis plays a major role in mediating cellular toxicity by CT. The role of endocytosis in mediating CT intoxication is not entirely clear; however, endosomal processing appears to involve CT migration to the Golgi and possibly other membrane-lined regions of the target cell cytoplasm. It now appears that endocytosis allows CT to encounter environmental stimuli that influence the course of cellular toxicity. The precise nature of these signals remains to be clearly established, but temperature sensitivity and pH have been suggested to be important in CT intoxication of rat hepatocytes and lung epithelial cell lines and may influence the cytoplasmic routing of CTB. Moreover, low pH has been suggested to have a role in cellular toxicity by other AB_5 toxins.

The mechanisms responsible for CTA1 transport are important because, in addition to its well-known toxic effect, CT possesses potent mucosal adjuvant properties that can be exploited in oral and other immunization strategies.

Production of mucosal vaccines using CT: previous strategies

One strategy to develop mucosal vaccines has been to employ bacterial enterotoxins such as cholera toxin (CT) from *Vibrio cholera* and the related *E. coli* heat-labile enterotoxin (LT), which are highly immunogenic when delivered mucosally and which can act as carriers to potentiate responses to non-related antigens. Both biotoxins are composed of an ADP-ribosylating A subunit which mediates toxicity, linked to a cyclic pentamer of B subunits which interacts specifically with monosialoganglioside (GM-1) sugars expressed on eukaryotic cells. During a natural infection, the function of the B subunits is to facilitate the binding and entry of the attached A subunit into GM-1-expressing cells. Although the holotoxins are potent adjuvants for a range of antigens and can modulate different immune functions including antigen presentation, cytokine production, and B-cell switching, their practical use is limited by the toxicity of the A subunit.

Attempts to diminish toxicity through mutations to the A subunit have raised considerable debate over the direct relationship between the toxicity and adjuvanticity of this subunit. If successful, these adjuvants would be relatively simple to formulate. Other attempts to solve the toxicity problem have included an attempt to develop non-toxic subunits as adjuvants. While vaccines incorporating B subunits appear to enhance the toxin's adjuvant activity by diminishing the toxin dosages required, B subunits have failed to gain popularity. Many investigations, however, only consider the B subunits as adjuvants, rather than as vehicles for antigen delivery.

Recent evidence indicates that B subunits might effectively serve to deliver antigen to the mucosal immune system when physically coupled to that antigen. In such case, the B subunit is known to significantly enhance the mucosal immune response to the carried antigen.

It is assumed that some of the adjuvant properties of LTB and CTB can be explained by the increased uptake of B subunit across GM-1-expressing epithelial cells lining mucosal tracts, thereby enhancing the amount of antigen passively delivered to the MALT inductive sites and the subsequent stimulation of antigen-specific B- and T-lymphocytes. LTB can also modulate immune activities such as cytokine production, lymphocyte apoptosis, and expression of B-cell activation molecules. These properties may also add to their effectiveness as adjuvants. What is clear is that the highly immunogenic nature of LTB and CTB and their ability to modulate lymphocyte activity is dependent upon their ability to bind GM-1.

Antigens can be physically attached to B5 subunits by either genetic or chemical means. Genetic fusion of epitopes to LTB has been successful in some cases, but genetic coupling of heterologous epitopes can interfere with the structure, secretion, GM-1-binding and immunogenicity of the LTB fusion proteins. Also, there are limitations to the size and type of antigen that can be attached to LTB by genetic fusion. An alternative and more flexible approach is chemical coupling, which may be used to attach larger and a wider range of antigens, including polysaccharides. CTB has been chemically coupled to antigens such as whole Sendai virus and purified AgI/II from *Streptococcus mutans* and these conjugates have been shown to stimulate strong immune responses at mucosal sites (gut, respiratory tract, genital tract) and in the circulation.

O'Dowd et al., 1999, Vaccine 17, pp. 1442-1453 generated a panel of LTB fusion proteins in which individual amino acids within an epitope tag were replaced with cysteine. Horseradish peroxidase (HRP) was chemically coupled to these cysteine residues. Following intranasal administration, high titer mucosal and systemic antibody responses to HRP were generated when HRP was chemically coupled to LTB. There was no antibody response when HRP was co-administered with LTB.

SUMMARY OF THE INVENTION

The invention provides novel sequence modifications to recombinant CTB or other AB_5-type B5 subunit proteins through site directed mutations, deletions or additions. The invention also contemplates a method for producing said proteins which can be cross-linked chemically, hydrophobically, or genetically, or otherwise associated with an immunogen adjuvant, microcapsule, drug or immune response modifier in a manner in which the CTB product or other AB_5-type B5 retains ganglioside specificity. In one embodiment, the recombinant B subunit proteins are specifically altered so that chemical conjugation to an immunogen, drug or immune

response modifier occurs in a residue specific manner and can be attached without eliminating the ganglioside affinity of the resultant conjugate. The recombinant B subunit proteins can be expressed and isolated in large amounts. This allows for a conjugate that is more homogeneous in composition and is known to bind to the ganglioside with an acceptable affinity. The vaccine(s) derived from this conjugate thereby target the mucosal tissues and promote both systemic and mucosal immune responses. The preferred embodiment of this invention is particularly advantageous for the recruitment of immunity to the predominant site of infection for infectious diseases, most of which typically colonize mucosal tissue surfaces.

One embodiment of the invention is a novel plasmid construct of a B5 expression system in which a B5 protein is expressed at high levels. In one embodiment, a recombinant cholera toxin B (rCTB) subunit protein including at least one mutation, addition, or deletion of residues between positions 1-103 of CTB is disclosed. Advantageously, the alteration allows chemical conjugation of said recombinant protein without interference to the ganglioside binding of CTB. In one embodiment, the mutation diminishes the ability of rCTB to combine at that site with any compound, drug, immunogen, immunomodulatory molecule, adjuvant or other bioactive molecule. However, the compound can bind more specifically at another site and deliver these compounds to the mucosal epithelium. Preferably, the mutation, deletion or addition is to positions 1-103 of wild type CTB.

In a further embodiment, at least one mutation includes K23U, K34U, K43U, K62U, K63U, K69U, K81U, K84U, C86U or K91U; wherein "U" is any amino acid that fails to promote chemical modification at those sites or wherein "U" represents the deletion of the residue, and the deletion of the amino acid results in a rCTB protein with a reduced ability to be covalently modified at those sites.

In one embodiment, advantageously, the at least one mutation, deletion or addition facilitates rCTB to combine at the site of the mutation, deletion or addition with any compound, drug, immunogen, immunomodulatory molecule, adjuvant or other bioactive molecule and to deliver the complex to the mucosal epithelium. Advantageously, the mutation, deletion or addition is to positions 1-103 of wild type CTB.

A further embodiment of the invention is a recombinant cholera toxin B (rCTB) subunit protein including at least one mutation or deletion that reduces the number of potential chemical modification sites in the recombinant protein as compared to a wild type CTB protein. Preferably, the mutation or deletion is at positions 1-103 of CTB. Advantageously, at least one mutation or addition increases the number of potential chemical modification sites in the recombinant protein at the mutation sites as compared to a wild type CTB protein. In one aspect of the invention, the mutations or additions are at positions 1-103 of CTB.

A further embodiment of the invention is a method of making a recombinant cholera toxin B (rCTB) subunit gene encoding a rCTB protein with a reduced number of modification sites as compared to a wild type CTB protein. The method includes providing a cholera toxin B (CTB) subunit gene encoding a CTB protein; selecting codons encoding amino acid residues favoring chemical modification of the CTB protein; and mutating or deleting the codons such that the resulting amino acid residues are unfavorable for chemical modification.

A further embodiment of the invention is a method of making a recombinant cholera toxin B (rCTB) subunit gene encoding a rCTB protein with an increase in chemical modification sites as compared to a wild type CTB protein. comprising providing a cholera toxin B (CTB) subunit gene encoding a CTB protein; selecting codons encoding amino acid residues unfavorable to chemical modification of the CTB protein; and mutating or adding said codons such that the resulting rCTB protein possesses favorable chemical modification capabilities; selecting codons encoding amino acid residues favorable to chemical modification of the CTB protein; and mutating or deleting said codons such that the resulting amino acids are incapable of chemical modification.

A further embodiment of the invention is a method of producing a recombinant cholera toxin B (rCTB) subunit gene encoding a rCTB protein with decreased chemical modification sites as compared to a wild type CTB protein, comprising providing a cholera toxin B (CTB) subunit gene encoding a CTB protein; selecting codons encoding amino acid residues unfavorable to chemical modification of the CTB protein; mutating or adding said codons such that the resulting rCTB protein possesses favorable chemical modification capabilities; selecting codons encoding amino acid residues favorable to chemical modification of the CTB protein; and mutating or deleting said codons such that the resulting amino acids are incapable of chemical modification.

A further embodiment of the invention is a method for producing a recombinant cholera toxin B (rCTB) subunit protein including the steps of obtaining a gene encoding an rCTB protein or mutated protein of the invention, adding a promoter, thereby producing an expression cassette; introducing the expression cassette into a suitable host cell, and cultivating the host cell under conditions whereby the expression cassette is translated into protein.

A further embodiment of the invention is a gene construct for producing a recombinant binding subunit protein of cholera toxin (CTB), having a promoter and a DNA sequence which encodes a rCTB protein operably linked in the proper reading frame.

A further embodiment of the invention is a method for producing a recombinant binding sub-unit protein of cholera toxin (CTB). The method includes expressing a gene construct in a suitable host cell and recovering CTB or mutations thereof.

A further embodiment of the invention is the creation of complexes of rCTB or variants thereof and compounds of whole or part, live, killed or reconstituted bacterium or virus, virus-like

particle, protein, peptide, glycoprotein, carbohydrate, polysaccharide, phospholipid, DNA, drug, biological response modifier, microparticles or adjuvant by genetic, covalent, hydrophobic or associative mechanisms.

A further embodiment of the invention is a gene fusion vector which includes a promoter, a DNA sequence encoding the cholera toxin binding subunit protein or mutant rCTB of the invention and an immunogenic peptide coding sequence, wherein the cholera toxin binding protein subunit and the immunogenic peptide coding sequences are operably linked in the proper reading frame, whereby a gene fusion protein results, and the gene fusion protein is expressed and secreted within inclusion bodies or medium.

A further embodiment of the invention is a method of generating an antibody or cellular immune response to an immunogen or immunogenic complex. Advantageously, the method includes providing the recombinant CTB protein of the invention, modifying the protein covalently with a heterobifunctional cross-linking reagent with a first and a second functional groups, wherein the first functional group is in chemical association with the recombinant CTB protein, covalently modifying the second functional group covalently with the immunogen, and administering the CTB protein modified in such a way that the animal generates an immune response.

A further embodiment of the invention is a method of generating an antibody or cellular immune response to an immunogen or immunogenic complex including providing the recombinant CTB protein of the invention, modifying the protein with a dimeric cross-linking reagent with a first and a second functional groups, wherein the first functional group is in chemical association with the recombinant CTB protein, covalently modifying the second functional group with an epitope, and administering the protein to the host until an immune response is generated.

A further embodiment of the invention is a method of generating an antibody or cellular immune response to an immunogen or immunogenic complex which includes providing the recombinant CTB protein of the invention, modifying the protein hydrophobically by prenylation or by covalent linkage in a CTB site directed manner to an amphipathic side group, mixing with an immunogenic complex containing any lipid or phospholipid micellar formulation, and administering the CTB-immunogen to the host until an immune response is generated.

A further embodiment of the invention is an rCTB expression vector including an MS-0 vector, and SEQ ID NO:1. The vector may be pML-CTBtac1.

A further embodiment of the invention is a method for generating an immune response to an immunogen comprising providing a recombinant AB_5 toxin B protein, prenylating the toxin B protein, mixing the prenylated toxin B protein with an immunogen to produce a hydrophobically coupled protein, and administering the protein to an animal to generate an antibody or cellular response. In one embodiment, the AB_5 toxin B protein may be cholera toxin B protein (CTB), *E.*

coli heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, or pertussis toxin B protein. Preferably, the AB_5 toxin B protein is cholera toxin B protein (CTB).

A further embodiment of the invention is a method of making a recombinant AB_5 toxin B (rB) subunit gene encoding a rB protein with an enhanced number of modifications sites as compared to the wild type rB protein, comprising providing a B subunit gene encoding a B protein, selecting codons encoding amino acid residues not involved in covalent modification of the B protein, and mutating said codons such that the resulting rB protein possesses enhanced modification capabilities.

A further embodiment of the invention is a method of making a recombinant AB_5 toxin B (rB) subunit gene encoding a rB protein with altered chemical modification capabilities as compared to the wild type B protein, including providing a toxin B (B) subunit gene encoding a B protein, selecting codons encoding amino acid residues which favor covalent modification of the B protein, mutating the codons such that the resulting rB protein possesses enhanced chemical modification capabilities at that site, selecting codons encoding amino acid residues which favor chemical modification of the B protein; and mutating the codons such that the resulting amino acids do not favor chemical modification at that site. In one embodiment, the rB protein is cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, or pertussis toxin B protein. In a further embodiment, the rB protein is cholera toxin B protein (CTB). Advantageously, the mutations include changing lysine, histidine, arginine or cysteine residues to any other amino acid residues. In a further embodiment, the mutations include changing any amino acid to lysine, histidine, arginine or cysteine.

A further embodiment of the invention is a gene fusion vector comprising a promoter, a DNA sequence encoding an AB_5 toxin ganglioside binding subunit protein and an immunogenic peptide coding sequence, wherein said AB_5 toxin ganglioside binding protein subunit and said immunogenic peptide coding sequences are operably linked in the proper reading frame, whereby a gene fusion protein results; and said gene fusion protein is expressed and secreted within inclusion bodies or medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the translated coding sequence of the rCTB protein produced by the MS-0 (Maxim Secretory -0) genetic expression vector (SEQ ID NOs:1 and 2). The encoded protein is identical to the wild-type sequence from *V. cholera* strain 569B.

Figure 2 is a plasmid map illustrating the MS-0 plasmid.

Figure 3 is the amino acid sequence of rCTB (SEQ ID NO:2), further illustrating the predicted secondary structure, position of lysine residues, and regions involved in binding to GM-1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention contemplates introducing mutations, such as amino acid additions or deletions, throughout the nucleic acid sequence of the B subunits from the family of AB_5 toxins (hereinafter AB_5 B subunit proteins) to produce mutant proteins that possess enhanced conjugation characteristics. The term "enhanced conjugation characteristics" refers to an AB_5 B subunit protein that can be conjugated by chemical or other means to drugs, immunogens, cytokines or other bioactive compounds such that the conjugated compound possesses increased bioactivity. Specifically, enhanced conjugation characteristics in an AB_5 B subunit protein results from the mutations taught here that are designed to decrease steric hinderance or the formation of inappropriate quaternary structures as a consequence of the process of conjugation. In this way, these mutations maximize the availability of the ligand binding sites on the mutant AB_5 B subunit proteins an conjugates composed thereof. One benefit of using such mutant AB_5 B subunit proteins is to improve the yield of bioactive structures following conjugation.

The invention describes mutants of AB_5 B subunit proteins comprising single or multiple amino acid substitutions, deletions, or insertions, throughout its sequence that result in novel, predictable spatial orientations of the molecules to be conjugated relative to one another. The invention further contemplates mutant AB_5 B subunit proteins with enhanced or altered conjugation characteristics that enhance the bioactivity of the resulting conjugate. Such a mutant AB_5 B subunit protein coupled to an immunogen or other bioactive compound serves to enhance the compound's activity in a subject to whom the mutant protein conjugate is administered.

AB_5 toxins consist of hexameric assemblies comprising a single catalytically active A-subunit and a pentamer of B-subunits. The B-pentamer of these toxins is capable of target cell recognition and binding even in the absence of the A-subunit. The toxin pentamers bind to saccharides, either to the olgosaccaride moieties of gangliosides in the cell membrane or to glycosylated proteins at the cell surface. The class of AB_5 toxins may be subdivided into families on the basis of sequence homology and catalytic activity. One family, the cholera toxin (CT) family includes, in addition to CT itself, the *E. coli* heat-labile enterotoxins LT and LT-II, and a less well-characterized toxin from *Campylobacter jejuni*. Another family, the shiga toxin family, comprises a number of toxins from *Shigella dysenteriae* and the shiga-like toxins (also known as verotoxins) from *E.coli*. Other AB_5 toxins include pertussis toxin from *B. pertussis* and the eukaryotic toxin ricin.

The AB_5 toxin LTB illustrates one relationship between this family of proteins. LTB shares extensive homology with CTB. The two toxins share 80% amino acid sequence identity, similar receptor specificity, catalytic activity, and immunological properties. Those AB_5 proteins with little sequence identity such as the shiga and pertussis toxins still share a high degree of structural similarity. This was determined based on the structure of the toxins in complex with the pentasaccharide from the toxin receptor and based on crystallographically determined details of toxin-receptor binding interactions (see Merritt et al. *Current Opinion in Structural Biology*, 1995; 5:165-171). Therefore, although other B5 subunit proteins can be distinct in amino acid sequence, they still possess comparable functional specificity. Some B5 holotoxins of this type are LT type IIa and IIb. Additionally, Shiga toxin, shiga-like toxin and Pertussis toxin share a similar receptor specificity of B5 subunits. Further, mutations such as those set out herein for rCTB, to these subunits would create delivery molecules enhanced in their ability to bind other bioactive compounds but which still retain ganglioside specificity. The concepts taught in this application for rCTB may be applied to all examples of B5 subunits, some of which are shown below in Table 1.

TABLE 1: AB_5 TOXIN SEQUENCES

AB5 family	Protein	NCBI accession #	SEQ ID NO.
CTB			
	CTB	GI:209555	32
		GI:758351	4
		GI:1827850	5
		GI:808900	6
		GI:229616	7
		GI:998409	8
		GI:2144685	9
		GI:1421511	10
	CTB classic (596B)	GI:48890	11
	CTB Ogawa 41	GI:2781121	12
	CTB Ogawa 41 R35D	GI:1421525	13
LTB			
	Classic LTB	GI:3062900	15
		GI:1169505	16

AB5 family	Protein	NCBI accession #	SEQ ID NO.
		GI:1395122	17
		GI:145833	18
	LT87	GI:1648865	19
		GI:223254	20
		GI:408996	21
		GI:494265	22
		GI:69630	23
	LT-IIa	GI:146671	24
	LT-IIb	GI:576584	25
Shiga toxin	Shiga toxin beta subunit	GI:152784	26
Shiga-like	Shiga-like toxin from *E. coli*	GI:4877349	27
Pertussis	Pertussis toxin chain S2	GI:144070	28
	S3	GI:144070	29
	S4	GI:144070	30
	S5	GI:144070	31

In many cases several toxin variants that differ in amino acid sequence have been characterized. The first CTB sequence shown (SEQ ID NO: 33) is the original sequence published by Sanchez and Holmgren that is identical to the one used in this application, except for the amino terminus. (SEQ ID NO:2). The first LTB sequence (SEQ ID NO:15) is derived from the sequence of the cloned gene. The other sequences listed in the table are variants of either CTB or LTB found in the NCBI gene bank. The list is not intended to show all variants, but to demonstrate the natural variation among these toxins.

Polynucleotides Encoding Mutant AB_5 B Subunit Proteins

The invention also relates to nucleic acids molecules comprising polynucleotide sequences encoding mutant AB_5 B subunit proteins, fragments or derivatives thereof. In one embodiment, the AB_5 B subunit proteins, fragments or derivatives contemplated for use with the invention possess mucosal tissue binding or association capabilities. These sequences generally contain at least one base insertion, deletion or substitution, or combinations thereof that result in single or multiple amino acid addition, deletion and substitution relative to the wild type AB_5 B subunit protein.

Due to the degeneracy of nucleotide coding sequences, any other DNA sequences that encode the same amino acid sequence for a mutant subunit may be used in the practice of the invention. These include but are not limited to nucleotide sequences comprising all or portions of the coding region of a AB_5 B subunit which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change.

AB_5 B Subunit Gene Cloning

Polynucleotides encoding AB_5 B subunits can be obtained by standard procedures from sources of cloned DNA, as would be represented by a "library" of biological clones, by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA purified from a desired cell type. Methods useful for conducting these procedures have been detailed by Sambrook et al., in *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); by Glover, D.M. (ed.), in *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K. (1985); and by *Current Protocols in Molecular Biology*, Eds. Ausubel, et al., John Wiley & Sons, Inc. (1998). The polymerase chain reaction (PCR) can be used to amplify sequences encoding an AB_5 B subunit in a genomic or cDNA library. Synthetic oligonucleotides can be utilized as primers in a PCR protocol using RNA or DNA, or a cDNA library, as a source of target sequences. The nucleic acid sequence being amplified can include cDNA or genomic DNA from any AB_5 B toxin producing bacterium. After successful isolation or amplification of a polynucleotide encoding a segment of an AB_5 B subunit, that segment can be molecularly cloned and sequenced, and utilized as a probe to isolate a complete cDNA or genomic clone. This, in turn, will permit characterization of the nucleotide sequence of the AB_5 B subunit-encoding polynucleotide, and the production of the AB_5 B subunit protein product for functional analysis and/or therapeutic or diagnostic use.

Alternatives to isolating the coding regions for the subunits include chemically synthesizing the gene sequence itself from the published sequence. Other methods are possible and within the scope of the invention. The above-methods are not meant to limit performance of the methods by which mutants of the B_5 subunits may be obtained.

The identified and isolated polynucleotide can be inserted into an appropriate cloning vector for amplification of the gene sequence. A large number of vector-host systems known in the art may be used for this purpose. Possible vectors include plasmids or modified viruses. Of course, the vector system must be compatible with the host cell used in these procedures. Such vectors include bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the pBLUESCRIPT vector (Stratagene, La Jolla, CA). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment of interest into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically

modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and mutant subunit gene may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection or electroporation so that the copies of the vector containing the gene sequence of interest is increased.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionation, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that comprise the mutant subunit gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the AB_5 B subunit-encoding polynucleotide may be obtained in large quantities by growing transformed host organisms, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA. Copies of the gene are used in mutagenesis experiments to study the structure and function of mutant AB_5 B subunit proteins.

Mutagenesis

The mutations present in mutant AB_5 B subunits, fragments and derivatives of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned coding region of the subunits can be modified by any of numerous strategies known in the art (see Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The polynucleotide sequence can be cleaved at appropriate sites using restriction endonucleases, followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a mutant subunit, care should be taken to ensure that the modified gene remains within the same translational reading frame, uninterrupted by translational stop signals in the gene region where the subunit is encoded.

Additionally, the polynucleotide sequence encoding the subunits can be mutated *in vitro* or *in vivo*, to create variations in coding regions (*e.g.* amino acid substitutions), and/or to create and/or destroy translation, initiation, and/or termination sequences, and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), PCR-based overlap extension (Ho et al., 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar et al., 1990,

Biotechniques, 8:404-407), or similar methods. The presence of mutations can be confirmed by double stranded dideoxy DNA sequencing.

Manipulations of the mutant subunit sequence may also be made at the protein level. Included within the scope of the invention are mutant AB_5 B subunits that are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; or metabolic synthesis in the presence of tunicamycin.

In addition, mutant AB_5 B peptide sequences or chemical analogs can be synthesized. For example, a peptide corresponding to a portion of a mutant subunit which comprises the desired mutated domain can be synthesized using an automated peptide synthesizer. Optionally, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the mutant subunit sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Expression of Mutant AB_5 B Subunit-Encoding Polynucleotides

The polynucleotide sequence encoding a mutant subunit of CTB or a functionally active fragment or other derivative thereof can be inserted into an appropriate expression vector. In the context of the invention, appropriate expression vectors will contain the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals may also be supplied by the native AB_5 B subunit cDNA or gene, and/or genomic sequences flanking the subunit gene. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include established or experimental bacterial expression systems; mammalian cell systems infected with a recombinant virus such as a vaccinia virus or adenovirus, insect cell systems infected with a virus such as a recombinant baculovirus, and microorganisms such as yeast containing vectors capable of replication in yeast.

The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements

may be used. In specific embodiments, a mutant subunit coding region or a sequence encoding a mutated and functionally active portion of the respective mutant subunit is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA synthetic techniques. Expression of polynucleotide sequences encoding mutant AB_5 B subunits or peptide fragments thereof may be regulated by a second polynucleotide sequence so that the mutant subunit(s) or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a mutant AB_5 B subunit or peptide fragments thereof may be controlled by any promoter/enhancer element known in the art. Promoters which may be used include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci.* U.S.A. 78:1441-1445), and the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42).

In a specific embodiment, a vector is used that comprises a promoter operably linked to the coding region of a mutant AB_5 B subunit, and one or more selectable markers (*e.g.*, an antibiotic resistance gene).

A host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers. In this matter, expression of the genetically engineered mutant subunits may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

Once a recombinant host cell which expresses the mutant subunit gene sequence(s) is identified, the gene product(s) can be purified and analyzed. The analysis is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay or other techniques useful for detecting the biological activity of the mutant subunit.

Production of Antibodies to Mutant Subunits and Analogs Thereof

According to the invention, mutant AB_5 B subunits, fragments or other derivatives thereof, alone or conjugated to an immunogenic molecule or some other protein of interest may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a mutant AB_5 B subunit, such as CTB, are produced. In another embodiment antibodies to a domain of a mutant subunit are produced. In another embodiment, antibodies to the immunogen are produced.

Various procedures known in the art may be used for the production of polyclonal antibodies directed against mutant AB_5 B subunits, fragments, or other derivatives thereof. For the production of antibodies, various host animals can be immunized by injection with the mutant subunits produced with the methods described herein. Appropriate host animals include rabbits, mice, rats, other mammals, as well as birds such as chickens. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, caprylic acid glycerides, peptides, polyoxyethylene sorbitan monoesters, oil and water emulsions, liposomes, poly-L-lactide co-glycolide (PLG) microspheres, protein carriers such as keyhole limpet hemocyanin or diphtheria toxin mutants, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed against mutant AB_5 B subunits, its fragments, or other derivatives thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture can be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). Human antibodies can be used and can be obtained by using human hybridomas (Cote et al., 1983, *Proc. Natl. Acad. Sci.* U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96). In fact, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci.* U.S.A. 81:6851-6855; Neuberger et al., 1984, *Nature* 312:604-608; Takeda et al., 1985, *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule specific for the epitope together with genes from a human antibody

molecule of appropriate biological activity can be used. The antibody products of these techniques fall within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce specific single chain antibodies against AB_5 B subunits, fragments, or derivatives thereof. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule, the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished using standard techniques known in the art. For example, the ELISA (enzyme-linked immunosorbent assay) would be an appropriate screening technique. For example, to select antibodies which recognize a specific domain of a mutant subunit, one may assay hybridomas for a product which binds to a fragment of a mutant subunit containing such domain. For selection of an antibody that specifically binds a mutant AB_5 B subunit, but which does not specifically bind the wild type protein, one can select on the basis of positive binding to the mutant and a lack of binding to the wild type protein. Antibodies specific for a domain of a mutant AB_5 B subunit, or fragment thereof are also provided by the invention.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the mutant AB_5 B subunits of the invention. These methods can involve labeling of the proteins, measuring levels thereof in appropriate physiological samples in diagnostic methods.

Structure and Function Analysis of Mutant AB_5 B Subunits

Described herein are methods for analyzing the structure and function of mutant AB_5 B subunits and for analyzing the *in vitro* activities and *in vivo* biological functions of the foregoing.

Once a mutant AB_5 B subunit is identified, it can be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique useful for purifying proteins. Functional properties of the protein can be evaluated using any suitable assay, including immunological, immunochemcial, biochemical and biological assays.

Alternatively, once a mutant AB_5 B subunit produced by a recombinant host cell is identified, the amino acid sequence of the subunit(s) can be determined using standard techniques for protein sequencing, including the use of an automated amino acid sequencer.

The functional activity of a mutant AB_5 B subunit, derivatives and fragments thereof can be assayed by various methods known in the art. For example, where a mutant AB_5 B subunit or fragment thereof is assayed for its ability to bind or compete with the corresponding wild type AB_5 B subunit. Alternatively, when AB_5 B subunits are assayed for antibody binding, various immunoassays known in the art can be used. These immunoassays include competitive and non-competitive assay systems using techniques such as radio-immunoassays, ELISA, "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays. Antibody binding can be detected by detecting a label on the primary antibody. Alternatively, the primary antibody can be detected by detecting binding of a secondary antibody or reagent to the primary antibody, particularly where the secondary antibody is labeled.

One of ordinary skill in the art may use the various assays discussed above to measure the binding affinity of a mutant AB_5 B subunit for its target ligand. Binding affinities for the mutant AB_5 B subunits may be reduced by one, two, three, four, or five, orders of magnitudes. A mutant AB_5 B subunit protein remains acceptable so long as the binding affinity of the mutant protein does not eliminate delivery of the mutant protein complex *in vivo*. The binding affinity of wild type CTB for Gm-1 has been calculated at approximately 4.6×10^{-12} as determined by BIAcor analysis (Duziemdo, et al. 1996, *Biochem.* 35:6375-6384). Mutants of CTB with altered modification characteristics may demonstrate reduced binding affinities for GM-1, however, wild type affinity is so strong that significant reductions of affinity following mutation may not reduce the *in vivo* delivery of the mutant protein complexes. Accordingly, mutant CTB proteins with binding affinities of approximately 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} are considered acceptable.

Mutant AB_5 B subunit proteins as vaccines

Mutant AB_5 B subunit proteins can be used as vaccines. When used in this manner, a mutant AB_5 B subunit may be coupled to an immunogen of choice. The mutant AB_5 B subunit proteins described herein are combined in a desired weight ratio so as to produce a vaccine that can elicit an optimal protective mucosal and systemic immune response from an immunized subject. In one embodiment, the mutant AB_5 B subunit and the immunogen component are mixed in a ratio in which the proportion of carrier component exceeds that of the immunogen, for example, at a

molecular weight ratio of about 500,000:1. In another embodiment of the invention, the mutant AB_5 B subunit and the immunogenic component are mixed in a molecular weight ratio of about 100,000:1. In still another embodiment the mutant AB_5 B subunit and the immunogen are mixed in a molecular weight ratio of about 1000:1, or 100:1, or 10:1. And in yet another embodiment, the molecular weight ratio can be that of 1:1. Alternatively, the components of the vaccine can also be combined in a ratio where the proportion of carrier component is less than that of the immunogen. For example, small compounds or peptides conjugated to a mutant rCtB described herein can be conjugated at a ratio of 1 rCtB homopentamer to 5, 10 or more immunogenic molecules.

Cross-linking reagents for use with mutant AB_5 B subunits

There are a variety of cross-linking procedures and reagents available to conjugate bioactive compounds, such as immunogens and vaccines to mutant AB_5 B subunits. Cross-linking procedures generally employ bifunctional reagents that modify amino acid side-chains. Typical amino acids modified include cysteine, lysine, and histamine, although any modifiable amino acid is contemplated for use with the invention. Bifunctional reagents are often classified on the basis of (i) chemical specificity; (ii) length of cross-bridge formed; (iii) whether the compound is a homo-bifunctional or hetero-bifunctional compound; (iv) whether the groups are chemically or photochemically reactive; and (v) whether the reagents contain a cleavable bond.

Frequently, cross-linking reagents are reactive with amino- groups or sulphydryl- containing side chains of amino acids, for example, as with lysine or cysteine side chains. The choice of a cross-linking reagent will depend on the target amino acid in both the ligand to be cross-linked and the target AB_5 B subunit. Cross-linking reagents for use with the invention modify target amino acid residues, produce a cross-link between the immunogen of choice and a mutant AB_5 B subunit that produces an immunogenic product, and does not substantially reduce the ligand binding affinity of the mutant AB_5 B subunit. An example of a cross-linking technique for lysine and cysteine residues is shown in Example 5.

A number of reagents are available to cross-link proteins. Examples of suitable cross-linking reagents include cleavable and non-cleavable cross-linking reagents. Some suitable cleavable cross-linking reagents that primarily react with primary amines such as lysine side chains include: dimethyl-3,3'-dithiobispropionimidate (DTBP), 2-iminothiolane (2-IT), *N*-succinimidyl-(4-azidophenyl)-1,3-dithiopropionate (SADP), ethyl-4-azidophenyl-1,4-dithiobutyrimidiate (EADB), 1-[*n*-2-hydroxy-5-azidobenzoyl)-2-aminoethyl]-4-(*N*-hydroxysuccinimidyl)-succinate (HAHS), and *N*-[-4-*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N*'-oxysulphosuccinimide ester. A suitable non-cleavable cross-linking agent that is specific for primary amines is succinimidyl trans-4-(maleimidylmethyl) cyclohexane-1-carbodylate (SMCC). Examples of suitable cleavable cross-

linking reagents that are specific for cysteine side chains include: *N*-(4-azidophenyl)phthalimide (APTP), *N*-[4-(*p*-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide (APDP), *N,N'*-bis(4-azidobenzoyl)cystine (ABC)2, and *N*-(4-azidobenzoylglycl)-*S*-(2-thiopyridyl)cysteine (AGTC).

Cross-linking with mutant AB_5 B subunit proteins can be induced with an aldehyde. For example, suitable aldehydes include glutaraldehyde, formaldehyde, glyceraldehyde, acetaldehyde, phenylaldehyde, valeraldehyde, or 3,4-dihydroxyphenylacetaldehyde. In another aspect of the invention, ketones can be used to modify and cross-link the vaccine components. Suitable ketones would include acetone, methyl ethyl ketone, 3-pentanone, or any other ketone known to one of ordinary skill in the art. Use of other cross-linking or conjugation agents is also contemplated, including *N*-succinimidyl 3-[2-pyridyldithio] propionate (SPDP), ultraviolet cross-linking and other protein cross-linking methods known in the art.

Mutants of AB_5 B subunit proteins

Three-dimensional structural analysis

A number of mutational analysis studies have been conducted to understand the component residues involved in either the ganglioside receptor or the toxic activity of AB_5 toxins. Mutations to the A subunit of LT have attempted to eliminate toxicity while maintaining adjuvant activity. The toxicity of CT and LT requires the binding of the toxin to a cell through the B subunit and activation of the toxic A1 subunit by cleavage from the A2 subunit. Mutations to the A subunit either prevent cleavage of the toxic A1 from the A2 subunit or alter the active residues of the A1 enzymatic site. Numerous and varied mutations of LTA at residues A7, S61, Q112, G118, A146 or A192 have been claimed to retain adjuvant activity concurrent with the loss of toxic activity (Komase et al. 1998 *Vaccine* 16:248-254). The adjuvant activity of many of these mutants is enhanced however, by the addition of rLTB (Verweij et al. 1998. *Vaccine* 16:2069-2076.)

Mutations to the ganglioside binding sites of the B subunits of the AB_5 toxins might adversely affect the ganglioside affinity characteristics of the protein. In fact, several mutations to CTB subunit residues influence receptor binding. For example, the mutation G33D ablates ganglioside affinity yet appears to retain polyglycosylceramide affinity (Backstrom et al. 1997 *Mol Microbiol* 24:489-497). Surprisingly, G33R mutations retain ganglioside affinity (Merritt et al. 1995 *Structure* 3:561-570).

Alternative substitutions may have surprising effects on the quaternary structure of CTB. For example, G35D substitutions diminish homopentamer formation (Merritt et al. 1995. *Structure* 3:561-570). Further, an affinity of the CTB residues between 10-14, particularly H13, for the ganglioside receptor of CTB has been suggested to lead to pentamer-pentamer formations (Merritt et al. 1995. *Structure* 3:561-570). His 13 has also been crucially implicated in the affinity of rCTB for Zn^{+2} (Dertzbaugh et al. 1998 *Prot Eng* 11: 577-581). In contrast, substitution of several novel peptide

sequences for positions 55-64 failed to influence ganglioside affinity (Backstrom et al. 1995. *Gene* 165:163-171).

In another example, site specific mutagenesis of the B subunit of LT-IIa enterotoxin at positions T13 and T14 significantly diminished GD1a ganglioside affinity. Similarly, mutagenesis of the LT-IIb positions T13, T14 and additionally T34 diminished ligand affinity. Interestingly, only serine substitutions at these sites allowed the retention of ligand affinity (Connell and Holmes. 1995. *Mol Microbiol* 16:21-31). Thus, mutation of residues known to be involved with ligand affinity may still be accomplished without loss of activity. Conversely, mutations (insertions, deletions, substitutions) of residues not known to be involved with ligand affinity may influence receptor activity. Site directed mutational analysis must therefore be conducted on a site by site basis.

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Structural Features of CTB

Amino acids 2-7 form a loop on the outer surface of rCTB. Residues Gln 3, Asn 4, Thr 6, and Asn7, all possess polar side chains which face out from the molecule. These side chains can be replaced with another amino acid to introduce a chemically modifiable amino acid side chains into this region. Residues 10-14 are reported to form an amino acid sequence with ganglioside receptor affinity. Mutations to this site, particularly H13, might create a more hydrophobic rCTB (Merritt et al. 1995. *Structure* 3:561-570). Amino acids 14-20 are thought to form a β-sheet on the outer surface of rCTB with many of the side chains facing outward. Among these amino acids are many polar side chain groups, particularly His 18, which may be amenable to lysine additions.

Residues 21-25 of CTB form a surface loop on the surface of rCTB. The loop is comprised of Asn 21, an uncharged polar amine, Asp 22, an acidic side chain, and Lys 23, a basic amine. Each side group is thought to face outward from the surface of the protein. The orientation of this residue suggests that it may play some role in structural stability of the monomer. Amino acid residue Phe 25 also faces outward from the molecular surface with its uncharged, nonpolar benzyl ring and might be mutated to introduce a modifiable amino acid into the sequence of the protein. Residues 25-30 form a β-pleated sheet within the CTB monomer that is not accessible to the molecular surface. Mutations to this site might be expected to disrupt the quaternary structure of rCTB. Residues 31-35 are referenced within the specification with regard to the Gm-1 receptor. Residues 36-40 of CTB are thought to form an internal β-pleated sheet as in 25-30.

Residues 41-44 form a surface exposed loop containing Lys 43 next to Asn 44. Asn 44 may be a good candidate for lysine due to its location, charge, and side chain similarity to Lys 43.

Residues 45-55 are thought to form a β-pleated sheet within the monomer. Residues 55-64 are thought to form an insert permissive site. Lysines at positions 62 and 63 within this region may be deleted or mutated without apparent adverse effect on the ganglioside binding affinity of rCTB mutants (Backstrom et al. 1995, *Gene* 165:163-171). Residues 60-78 form an internal hydrophobic α-helix in the homopentamer. Significant changes to this sequence, particularly to residues 65-78, might be expected to interfere with homopentamer formation.

Residues 79-85 of CTB form a loop on the surface of the CTB monomer. Within the loop, Lys 81 and Lys 84 face outward from the monomeric structure. Lys 81 and 84 would appear to be an optimal target residues for conjugation due to their position contralateral to the receptor and surface location within the monomer.

CTB residues 86-95 form internalized side chains and inferior loop contact points with Gm-1, encompass the Lys 91 successfully produced and claimed herein. CTB residues 96-103 form an internal sheet which ends with an optimally positioned Asn 103. This Asn 103 is a key target for Lys insertion due to its similar charge, structure and on the molecule.

In view of the structural analysis discussed above, it is clear that a large number of mutations can result in a mutant CTB protein with altered conjugation characteristics. Some mutants will preserve, enhance, or at least not detrimentally reduce the Gm-1 binding affinity of the mutant CTB proteins described herein. On the other hand, some loss of Gm-1 affinity is tolerable given the extraordinarily high initial affinity (4.6×10^{-12}) of the cholera toxin B for the Gm-1 receptor. Binding affinity of the mutant proteins can be determined using a number of assays well known in the art.

Mutants of Recombinant cholera Toxin Subunit B (rCTB)

The invention contemplates mutations, substitutions, deletions, or insertions of one, two, three, four or more amino acid residues in the amino acid sequence of cholera toxin subunit B. The full length amino acid sequence of wild type rCTB is shown in FIGURE 1 (SEQ ID NO: 2).

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: T1X, P2X, Q3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, H18X, T19X, L20X, N21X, D22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, G33X, K34X, R35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, N44X, G45X, A46X, T47X, F48X, Q49X, V50X, E51X, V52X, P53X, G54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

Lysine Substitutions in rCTB

Conjugation of rCTB to immunogens derived from infectious diseases can create vaccines which induce protection following mucosal delivery. Classic conjugation chemistry targets are reactive amino acid side chain groups such as primary amines, carboxylic acids, aldehydes, or sulfhydryls. The primary reactive amino acids in rCTB are lysines (see Figure 3). The B subunit of cholera toxin contains nine (9) lysine amino acid residues. A number of these residues are contemplated to play a role in facilitating conjugation of bioactive compounds to the rCTB protein. These lysine residues are distributed at various places throughout the CTB sequence. Those lysine

residues that are near the Gm-1 binding site (e.g. K91) can couple to a bioactive compound of interest in a conformation which interferes with ganglioside binding. Further, any lysine residues to which the bioactive compound is coupled may induce a conformational change upon conjugation, which prevents or disrupts the formation of the quaternary structure required for ganglioside binding. Accordingly, a number of mutant rCTB proteins with increased or decreased numbers of conjugation sites as compared to the wild type form of the protein are disclosed herein.

The present approach involves directing the covalent linkage of an AB_5 B subunit carrier and a bioactive molecule such that the bioactive molecule does not interfere with the ganglioside site of CTB. Site directed mutagenesis was employed to create a panel of mutations at Lys91 of the rCTB coding sequence. These mutations resulted in several mutants which produce levels of protein, *in vitro*, comparable to those of the wild type and which can effectively deliver bioactive CTB-immunogen complexes to the mucosal immune system.

Accordingly, one embodiment discussed herein articulates a number of mutant rCTB proteins with amino acid substitutions that replace the lysine residues found in the wild type rCtB protein. Of the 9 lysine residues present in wild type CTB, the invention contemplates recombinant CTB proteins with one or more of the following amino acid substitutions: K23X, K34X, K43X, K62X, K63X, K69X, K81X, K84X, and K91X; wherein "X" is any amino acid that results in a rCTB protein with a reduced ability to conjugate at the mutant sites. These mutants are also contemplated to maintain a substantial degree of Gm-1 binding affinity as compared to the wild type or unmutated form of the protein. Moreover, as mentioned before, some loss of affinity is allowable due to the extraordinarily high initial affinity (4.6×10^{-12}) of the wild type CTB for the Gm-1 receptor.

There are a number of lysine residues that are thought to result in particularly deleterious conjugation events given the proximity of these residues to the Gm-1 binding site. These residues include K34, K62, K63, and K91. Mutation of these residues favors conjugation events that will not interfere with Gm-1 binding affinity.

The invention further contemplates mutating the lysine residue at K69 to eliminate conjugation at this position. Lysine 69 is thought to be distal from the Gm-1 binding site; however, conjugation to this residue might reduce rCtB homopentamer self-assembly. Incomplete quaternary structure formation is known to diminish Gm-1 affinity and likely also diminishes the bioactivity of a rCTB conjugated molecule.

The remaining lysine residues in CTB are K23, K43, K81, and K84. Each of these residues are contemplated as acceptable sites for conjugation. One or more of these residues can be mutated to reduce the total number of sites for conjugation.

McCann et al. (Biochem. 1997, 36:9169) demonstrated that Lys91 was the most reactive of all lysine residues in CTB when reacted with "small" (fluorescent) molecular probes. When CTB

is bound to Gm-1, Lys91 is positioned proximal to the terminal galactose residue of Gm-1. Therefore, conjugation of large molecular weight bioactive molecules is likely to result in steric hinderance of the Gm-1 binding site of rCTB. Similarly, reaction of larger molecular weight compounds with Lys69, which is located within the hydrophobic core of the pentamer, would disfavor reformation of bioactive CTB homopentamers.

Lys91 possesses a basic amino side chain with which the hydroxylated galactose of Gm-1 may interact and facilitate the affinity of rCTB for Gm-1. A knockout of Lys91 might therefore be expected to significantly decrease the affinity of the mutant rCTB for Gm-1 and effect a decrease in recombinant production levels.

Lysine additions in rCTB

The invention further contemplates the addition of amino acid residues throughout the amino acid sequence of rCTB to direct conjugation of immunogens, immunomodulators, drugs, or other bioactive molecules, to specified sites within rCTB. Any residue of rCTB can be mutated to introduce an amino acid residue that promotes acceptable conjugation events. Examples of these mutations include Q3X, N4X, T6X, N7X, H18X, N21X, F25X, N44X, M101X, and N103X wherein "X" is any amino acid that results in the addition of an acceptable conjugation site. Specific examples of suitable amino acids for use at these sites include lysine and cysteine. Accordingly, one or more of the following mutations: Q3K, N4K, T6K, N7K, H18K, N21K, F25K, N44K, M101K, N103K, Q3C, N4C, T6C, N7C, H18C, N21C, F25C, N44C, M101C, and N103C are contemplated.

Alternative Chemical Cross-linking Moieties

A further approach to specific chemical coupling or site-directed chemical coupling is to create a site for chemical coupling using a cysteine residue rather than a lysine residue. Accordingly, the following cysteine insertions are created: H18C and M101C. The site-directed mutagenesis is performed as in Examples 1-2. Example 5 provides techniques for chemically coupling these residues to an immunogen of interest.

Mutants in the N-terminus of rCTB

The wild type amino acid sequence of rCTB is shown in Figure 1. The invention contemplates various mutations throughout the rCTB amino acid sequence. In one embodiment, one or more mutations are introduced into the N-terminus of the protein sequence. For example, in one embodiment, an alanine residue is introduced at the first position of the rCTB amino acid sequence. Introduction of this particular amino acid creates a defined signal sequence cleavage site, as opposed to the threonine residue at the amino terminus of the wild type form of rCTB. This cleavage site can be important in post-translational modifications.

Mutants of other CTB family members identified by NCBI accession number

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:758351 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutants include: M1X, I2X, K3X, L4X, K5X, F6X, G7X, V8X, F9X, F10X, T11X, V12X, L13X, L14X, S15X, S16X, A17X, Y18X, A19X, H20X, G21X, T22X, P23X, Q24X, N25X, I26X, T27X, D28X, L29X, C30X, A31X, E32X, S33X, H34X, N35X, T36X, Q37X, I38X, Y39X, T40X, L41X, N42X, D43X, K44X, I45X, F46X, S47X, Y48X, T49X, E50X, S51X, L52X, A53X, G54X, K55X, R56X, E57X, M58X, A59X, I60X, I61X, T62X, F63X, K64X, N65X, G66X, A67X, I68X, F69X, Q70X, V71X, E72X, V73X, P74X, S75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, A96X, Y97X, L98X, T99X, E100X, A101X, K102X, V103X, E104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, H115X, A116X, I117X, A118X, A119X, I120X, S121X, M122X, A123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI:758351 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:1827850 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutants include: T1X, P2X, Q3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, Y18X, T19X, L20X, N21X, D22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, G33X, K34X, R35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, N44X, G45X, A46X, I47X, F48X, Q49X, V50X, E51X, V52X, P53X, S54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, T87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 1827850 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:808900 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, I2X, K3X, L4X, K5X, F6X, G7X, V8X, F9X, F10X, T11X, V12X, L13X, L14X, S15X, S16X, A17X, Y18X, A19X, H20X, G21X, T22X, P23X, Q24X, N25X, I26X, T27X, D28X, L29X, C30X, A31X, E32X, Y33X, H34X, N35X, T36X, Q37X, I38X, H39X, T40X, L41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, L52X, A53X, G54X, N55X, R56X, E57X, M58X, A59X, I60X, I61X, T62X, F63X, K64X, N65X, G66X, A67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, A96X, Y97X, L98X, T99X, E100X, A101X, K102X, V103X, E104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, H115X, A116X, I117X, A118X, A119X, I120X, S121X, M122X, A123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI:808900 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:229616 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: T1X, P2X, E3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, H18X, T19X, L20X, N21X, N22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, G33X, K34X, R35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, D44X, G45X, A46X, T47X, F48X, E49X, V50X, E51X, V52X, P53X, G54X, S55X, E56X, H57X, I58X, D59X, S60X, E61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 229616 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:998409 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: T1X, P2X, Q3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, Y18X, T19X, L20X, N21X, D22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, G33X, K34X, R35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, N44X, G45X, A46X, I47X, F48X, Q49X, V50X, E51X, V52X, P53X, G54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 998409 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:2144685 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, I2X, K3X, L4X, K5X, F6X, G7X, V8X, F9X, F10X, T11X, V12X, L13X, L14X, S15X, S16X, A17X, Y18X, A19X, H20X, G21X, T22X, P23X, Q24X, N25X, I26X, T27X, D28X, L29X, C30X, A31X, E32X, S33X, H34X, N35X, T36X, Q37X, I38X, Y39X, T40X, L41X, N42X, D43X, K44X, I45X, F46X, S47X, Y48X, T49X, E50X, S51X, L52X, A53X, G54X, K55X, R56X, E57X, M58X, A59X, I60X, I61X, T62X, F63X, K64X, N65X, G66X, A67X, I68X, F69X, Q70X, V71X, E72X, V73X, P74X, S75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, A96X, Y97X, L98X, T99X, E100X, A101X, K102X, V103X, E104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, H115X, A116X, I117X, A118X, A119X, I120X, S121X, M122X, A123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI:2144685 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:1421511 without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: T1X, P2X, Q3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, H18X, T19X, L20X, N21X, D22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, D33X, K34X, R35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, N44X, G45X, A46X, T47X, F48X, Q49X, V50X, E51X, V52X, P53X, G54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 1421511 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:48890 (classic 569B) without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, I2X, K3X, L4X, K5X, F6X, G7X, V8X, F9X, F10X, T11X, V12X, L13X, L14X, S15X, S16X, A17X, Y18X, A19X, H20X, G21X, T22X, P23X, Q24X, N25X, I26X, T27X, D28X, L29X, C30X, A31X, E32X, Y33X, H34X, N35X, T36X, Q37X, I38X, H39X, T40X, L41X, N42X, D43X, K44X, I45X, F46X, S47X, Y48X, T49X, E50X, S51X, L52X, A53X, G54X, K55X, R56X, E57X, M58X, A59X, I60X, I61X, T62X, F63X, K64X, N65X, G66X, A67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, A96X, Y97X, L98X, T99X, E100X, A101X, K102X, V103X, E104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, H115X, A116X, I117X, A118X, A119X, I120X, S121X, M122X, A123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 48890 (classic 569B) with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:2781121 (Ogawa 41) without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, T2X, P3X, Q4X, N5X, I6X, T7X, D8X, L9X, C10X, A11X, E12X, Y13X, H14X, N15X, T16X, Q17X, I18X, H19X, T20X, L21X, N22X, D23X, K24X, I25X, F26X, S27X, Y28X, T29X, E30X, S31X, L32X, A33X, G34X, K35X, R36X, E37X, M38X, A39X, I40X, I41X, T42X, F43X, K44X, N45X, G46X, A47X, T48X, F49X, Q50X, V51X, E52X, V53X, P54X, G55X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid,. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 2781121 (Ogawa 41) with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rCTB GI:1421525 (Ogawa 41 R35D) without deleterious effect to the ganglioside (Gm-1) binding affinity of the recombinantly produced molecule. Such mutations include: T1X, P2X, Q3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, H18X, T19X, L20X, N21X, D22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, G33X, K34X, D35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, N44X, G45X, A46X, T47X, F48X, Q49X, V50X, E51X, V52X, P53X, G54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rCTB GI: 1421525 (Ogawa 41 R35D) with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its Gm-1 binding activity.

Mutants of LTB and LTB family members identified by NCBI accession number

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:3062900 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, C18X, A19X, Y20X, G21X, A22X, P23X, Q24X, S25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, K64X, S65X, G66X, A67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, E123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:3062900 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:1169505 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, Y18X, A19X, H20X, G21X, A22X, P23X, Q24X, T25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, K64X, S65X, G66X, E67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, K123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of

any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:1169505 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:1395122 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, C18X, A19X, Y20X, G21X, A22X, P23X, Q24X, S25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, K64X, S65X, G66X, A67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, M121X, E122X, N123X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:1395122 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:145833 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, Y18X, A19X, H20X, G21X, A22X, P23X, Q24X, T25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, K64X, S65X, G66X, E67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, K123X, N124X, wherein the first letter is the native amino acid appearing at the

position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:145833 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:1648865 (LT87) without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, F6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, C18X, A19X, H20X, G21X, A22X, P23X, Q24X, S25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, H34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, K64X, S65X, G66X, A67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, E123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI: 1648865 (LT87) with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:223254 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, Y18X, A19X, H20X, G21X, A22X, P23X, Q24X, T25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, M64X, S65X, G66X, E67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X,

D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, K123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:223254 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:408996 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, C18X, A19X, Y20X, G21X, A22X, P23X, Q24X, S25X, I26X, T27X, E28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, K64X, S65X, G66X, A67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, E123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:408996 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:494265 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: A1X, P2X, Q3X, T4X, I5X, T6X, E7X, L8X, C9X, S10X, E11X, Y12X, R13X, N14X, T15X, Q16X, I17X, Y18X, T19X, I20X, N21X, D22X, K23X, I24X, L25X, S26X, Y27X, T28X, E29X, S30X, M31X, A32X, G33X, K34X, R35X, E36X, M37X, V38X, I39X, I40X, T41X, F42X, K43X, S44X, G45X, E46X, T47X, F48X, Q49X, V50X, E51X, V52X, P53X, G54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, T75X, Y76X, L77X,

T78X, E79X, T80X, K81X, I82X, D83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X, T92X, P93X, N94X, S95X, I96X, A97X, A98X, I99X, S100X, M101X, K102X, N103X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:494265 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

The amino acid substitutions encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLTB GI:69630 without deleterious effect to the ganglioside and glycosphingolipid binding affinity of the recombinantly produced molecule. Such mutations include: M1X, N2X, K3X, V4X, K5X, C6X, Y7X, V8X, L9X, F10X, T11X, A12X, L13X, L14X, S15X, S16X, L17X, Y18X, A19X, H20X, G21X, A22X, P23X, Q24X, T25X, I26X, T27X, Q28X, L29X, C30X, S31X, E32X, Y33X, R34X, N35X, T36X, Q37X, I38X, Y39X, T40X, I41X, N42X, D43X, K44X, I45X, L46X, S47X, Y48X, T49X, E50X, S51X, M52X, A53X, G54X, K55X, R56X, E57X, M58X, V59X, I60X, I61X, T62X, F63X, M64X, S65X, G66X, E67X, T68X, F69X, Q70X, V71X, E72X, V73X, P74X, G75X, S76X, Q77X, H78X, I79X, D80X, S81X, Q82X, K83X, K84X, A85X, I86X, E87X, R88X, M89X, K90X, D91X, T92X, L93X, R94X, I95X, T96X, Y97X, L98X, T99X, E100X, T101X, K102X, I103X, D104X, K105X, L106X, C107X, V108X, W109X, N110X, N111X, K112X, T113X, P114X, N115X, S116X, I117X, A118X, A119X, I120X, S121X, M122X, K123X, N124X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid,. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLTB GI:69630 with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside and glycosphingolipid binding activity.

Mutants of LT-IIa

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLT-IIa NCBI Accession No. GI:146671 without deleterious effect to the ganglioside (GD1b, among others) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, S2X, S3X, K4X, K5X, I6X, I7X, G8X, A9X, F10X, V11X, L12X, M13X, T14X, G15X, I16X, L17X, S18X, G19X, Q20X, V21X, Y22X, A23X, G24X, V25X, S26X, E27X, H28X, F29X, R30X, N31X, I32X, C33X, N34X, Q35X, T36X, T37X, A38X, D39X, I40X, V41X, A42X, G43X, V44X, Q45X, L46X, K47X, K48X, Y49X, I50X, A51X, D52X, V53X, N54X, T55X, N56X, T57X, R58X, G59X, I60X, Y61X,

V62X, V63X, S64X, N65X, T66X, G67X, G68X, V69X, W70X, Y71X, I72X, P73X, G74X, G75X, R76X, D77X, Y78X, P79X, D80X, N81X, F82X, L83X, S84X, G85X, E86X, I87X, R88X, K89X, T90X, A91X, M92X, A93X, A94X, I95X, L96X, S97X, D98X, T99X, K100X, V101X, N102X, L103X, C104X, A105X, K106X, T107X, S108X, S109X, S110X, P111X, N112X, H113X, I114X, W115X, A116X, M117X, E118X, L119X, D120X, R121X, E122X, S123X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLT-IIa with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its GD1b binding activity.

Mutants of LT-IIb

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of rLT-IIb NCBI accession no. GI:576584 without deleterious effect to the ganglioside (GDIa among others) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, S2X, F3X, K4X, K5X, I6X, I7X, K8X, A9X, F10X, V11X, I12X, M13X, A14X, A15X, L16X, V17X, S18X, V19X, Q20X, A21X, H22X, A23X, G24X, A25X, S26X, Q27X, F28X, F29X, K30X, D31X, N32X, C33X, N34X, R35X, T36X, T37X, A38X, S39X, L40X, V41X, E42X, G43X, V44X, E45X, L46X, T47X, K48X, Y49X, I50X, S51X, D52X, I53X, N54X, N55X, N56X, T57X, D58X, G59X, M60X, Y61X, V62X, V63X, S64X, S65X, T66X, G67X, G68X, V69X, W70X, R71X, I72X, S73X, R74X, A75X, K76X, D77X, Y78X, P79X, D80X, N81X, V82X, M83X, T84X, A85X, E86X, M87X, R88X, K89X, I90X, A91X, M92X, A93X, A94X, V95X, L96X, S97X, G98X, M99X, R100X, V101X, N102X, M103X, C104X, A105X, S106X, P107X, A108X, S109X, S110X, P111X, N112X, V113X, I114X, W115X, A116X, I117X, E118X, L119X, E120X, A121X, E122X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant rLT-IIb with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside GDIa binding activity.

Mutants of Shiga toxin Beta subunit

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of Shiga toxin Beta subunit NCBI Accession No. GI:152784 without deleterious effect to the carbohydrate binding affinity of the recombinantly produced molecule for Gal alpha 1----4Gal beta 1 ----4Glc and globotrioside trisaccharides. Such mutations include: M1X, K2X, K3X, T4X, L5X, L6X, I7X, A8X, A9X, S10X,

L11X, S12X, F13X, F14X, S15X, A16X, S17X, A18X, L19X, A20X, T21X, P22X, D23X, C24X, V25X, T26X, G27X, K28X, V29X, E30X, Y31X, T32X, K33X, Y34X, N35X, D36X, D37X, D38X, T39X, F40X, T41X, V42X, K43X, V44X, G45X, D46X, K47X, E48X, L49X, F50X, T51X, N52X, R53X, W54X, N55X, L56X, Q57X, S58X, L59X, L60X, L61X, S62X, A63X, Q64X, I65X, T66X, G67X, M68X, T69X, V70X, T71X, I72X, K73X, T74X, N75X, A76X, C77X, H78X, N79X, G80X, G81X, G82X, F83X, S84X, E85X, V86X, I87X, F88X, R89X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant Shiga toxin Beta subunit with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its carbohydrate binding activity.

Mutants of verotoxin (Shiga-like toxin) beta subunit from *E. coli*

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of Shiga-like toxin Beta subunit from *E. coli* NCBI Accession No. GI:4877349 without deleterious effect to the carbohydrate binding affinity for globotriaosyl ceramide, Gb3 or blood group P1 antigenic trisaccharide of the recombinantly produced molecule. Such mutations include: M1X, K2X, K3X, I4X, F5X, V6X, A7X, A8X, L9X, F10X, A11X, F12X, V13X, S14X, V15X, N16X, A17X, M18X, A19X, A20X, D21X, C22X, A23X, K24X, G25X, K26X, I27X, E28X, F29X, S30X, K31X, Y32X, N33X, E34X, N35X, D36X, T37X, F38X, T39X, V40X, K41X, V42X, A43X, G44X, K45X, E46X, Y47X, W48X, T49X, N50X, R51X, W52X, N53X, L54X, Q55X, P56X, L57X, L58X, Q59X, S60X, A61X, Q62X, L63X, T64X, G65X, M66X, T67X, V68X, T69X, I70X, K71X, S72X, N73X, T74X, C75X, A76X, S77X, G78X, S79X, G80X, F81X, A82X, E83X, V84X, Q85X, F86X, N87X, N88X, D89X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant Shiga-like toxin Beta subunit from *E. coli* with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its carbohydrate binding activity.

Mutants of pertussis toxin B subunits

The amino acid substitutions contemplated in the invention encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of pertussis toxin S2 subunit NCBI Accession No. GI:144070 without deleterious effect to the ganglioside (sialoglycoproteins) binding affinity of the recombinantly produced molecule. Such mutations

include: M1X, P2X, I3X, D4X, R5X, K6X, T7X, L8X, C9X, H10X, L11X, L12X, S13X, V14X, L15X, P16X, L17X, A18X, L19X, L20X, G21X, S22X, H23X, V24X, A25X, R26X, A27X, S28X, T29X, P30X, G31X, I32X, V33X, I34X, P35X, P36X, Q37X, E38X, Q39X, I40X, T41X, Q42X, H43X, G44X, S45X, P46X, Y47X, G48X, R49X, C50X, A51X, N52X, K53X, T54X, R55X, A56X, L57X, T58X, V59X, A60X, E61X, L62X, R63X, G64X, S65X, G66X, D67X, L68X, Q69X, E70X, Y71X, L72X, R73X, H74X, V75X, T76X, R77X, G78X, W79X, S80X, I81X, F82X, A83X, L84X, Y85X, D86X, G87X, T88X, Y89X, L90X, G91X, G92X, E93X, Y94X, G95X, G96X, V97X, I98X, K99X, D100X, G101X, T102X, P103X, G104X, G105X, A106X, F107X, D108X, L109X, K110X, T111X, T112X, F113X, C114X, I115X, M116X, T117X, T118X, R119X, N120X, T121X, G122X, Q123X, P124X, A125X, T126X, D127X, H128X, Y129X, Y130X, S131X, N132X, V133X, T134X, A135X, T136X, R137X, L138X, L139X, S140X, S141X, T142X, N143X, S144X, R145X, L146X, C147X, A148X, V149X, F150X, V151X, R152X, S153X, G154X, Q155X, P156X, V157X, I158X, G159X, A160X, C161X, T162X, S163X, P164X, Y165X, D166X, G167X, K168X, Y169X, W170X, S171X, M172X, Y173X, S174X, R175X, L176X, R177X, K178X, M179X, L180X, Y181X, L182X, I183X, Y184X, V185X, A186X, G187X, I188X, S189X, V190X, R191X, V192X, H193X, V194X, S195X, K196X, E197X, E198X, Q199X, Y200X, Y201X, D202X, Y203X, E204X, D205X, A206X, T207X, F208X, E209X, T210X, Y211X, A212X, L213X, T214X, G215X, I216X, S217X, I218X, C219X, N220X, P221X, G222X, S223X, S224X, L225X, C226X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant pertussis toxin S2 subunit with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside (sialoglycoproteins) binding activity.

The amino acid substitutions encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of pertussis toxin S3 subunit NCBI Accession No. GI:144070 without deleterious effect to the ganglioside (sialoglycoproteins) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, L2X, I3X, N4X, N5X, K6X, K7X, L8X, L9X, H10X, H11X, I12X, L13X, P14X, I15X, L16X, V17X, L18X, A19X, L20X, L21X, G22X, M23X, R24X, T25X, A26X, Q27X, A28X, V29X, A30X, P31X, G32X, I33X, V34X, I35X, P36X, P37X, K38X, A39X, L40X, F41X, T42X, Q43X, Q44X, G45X, G46X, A47X, Y48X, G49X, R50X, C51X, P52X, N53X, G54X, T55X, R56X, A57X, L58X, T59X, V60X, A61X, E62X, L63X, R64X, G65X, N66X, A67X, E68X, L69X, Q70X, T71X, Y72X, L73X, R74X, Q75X, I76X, T77X, P78X, G79X, W80X, S81X, I82X, Y83X, G84X, L85X, Y86X, D87X, G88X, T89X, Y90X, L91X, G92X, Q93X, A94X, Y95X, G96X, G97X, I98X, I99X, K100X,

D101X, A102X, P103X, P104X, G105X, A106X, G107X, F108X, I109X, Y110X, R111X, E112X, T113X, F114X, C115X, I116X, T117X, T118X, I119X, Y120X, K121X, T122X, G123X, Q124X, P125X, A126X, A127X, D128X, H129X, Y130X, Y131X, S132X, K133X, V134X, T135X, A136X, T137X, R138X, L139X, L140X, A141X, S142X, T143X, N144X, S145X, R146X, L147X, C148X, A149X, V150X, F151X, V152X, R153X, D154X, G155X, Q156X, S157X, V158X, I159X, G160X, A161X, C162X, A163X, S164X, P165X, Y166X, E167X, G168X, R169X, Y170X, R171X, D172X, M173X, Y174X, D175X, A176X, L177X, R178X, R179X, L180X, L181X, Y182X, M183X, I184X, Y185X, M186X, S187X, G188X, L189X, A190X, V191X, R192X, V193X, H194X, V195X, S196X, K196X, E197X, E198X, Q199X, Y200X, Y201X, D202X, Y203X, E204X, D205X, A206X, T207X, F208X, Q209X, T210X, Y211X, A212X, L213X, T214X, G215X, I216X, S217X, L218X, C219X, N220X, P221X, A222X, A223X, S224X, I225X, C226X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant pertussis toxin S3 subunit with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside (sialoglycoproteins) binding activity.

The amino acid substitutions further encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of pertussis toxin S4 subunit NCBI Accession No. GI:144070 without deleterious effect to the ganglioside (sialoglycoproteins) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, L2X, R3X, R4X, F5X, P6X, T7X, R8X, T9X, T10X, A11X, P12X, G3X, Q14X, G15X, G16X, A17X, R18X, R19X, S20X, R21X, V22X, R23X, A24X, L25X, A26X, W27X, L28X, L29X, A30X, S31X, G32X, A33X, M34X, T35X, H36X, L37X, S38X, P39X, A40X, L41X, A42X, D43X, V44X, P45X, Y46X, V47X, L48X, V49X, K50X, T51X, N52X, M53X, V54X, V55X, T56X, S57X, V58X, A59X, M60X, K61X, P62X, Y63X, E64X, V65X, T66X, P67X, T68X, R69X, M70X, L71X, V72X, C73X, G74X, I75X, A76X, A77X, K78X, L79X, G80X, A81X, A82X, A83X, S84X, S85X, P86X, D87X, A88X, H89X, V90X, P91X, F92X, C93X, F94X, G95X, K96X, D97X, L98X, K99X, R100X, P101X, G102X, S103X, S104X, P105X, M106X, E107X, V108X, M109X, L110X, R111X, A112X, V113X, F114X, M115X, Q116X, Q117X, R118X, P119X, L120X, R121X, M122X, F123X, L124X, G125X, P126X, K127X, Q128X, L129X, T130X, F131X, E132X, G133X, K134X, P135X, A136X, L137X, E138X, L139X, I140X, R141X, M142X, V143X, E144X, C145X, S146X, G147X, K148X, Q149X, D150X, C151X, P152X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when

introduced in place of the wild type amino acid residue, results in a mutant or variant pertussis toxin S4 subunit with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside (sialoglycoproteins) binding activity.

The amino acid substitutions contemplated in the invention likewise encompass any such substitution, deletion, or insertion that selectively alters the conjugation characteristics of pertussis toxin S5 subunit NCBI Accession No. GI:144070 without deleterious effect to the ganglioside (sialoglycoproteins) binding affinity of the recombinantly produced molecule. Such mutations include: M1X, Q2X, R3X, Q4X, A5X, G6X, L7X, P8X, L9X, K10X, A11X, N12X, P13X, M14X, H15X, T16X, I17X, A18X, S19X, I20X, L21X, L22X, S23X, V24X, L25X, G26X, I27X, Y28X, S29X, P30X, A31X, D32X, V33X, A34X, G35X, L36X, P37X, T38X, H39X, L40X, Y41X, K42X, N43X, F44X, T45X, V46X, Q47X, E48X, L49X, A50X, L51X, K52X, L53X, K54X, G55X, K56X, N57X, Q58X, E59X, F60X, C61X, L62X, T63X, A64X, F65X, M66X, S67X, G68X, R69X, S70X, L71X, V72X, R73X, A74X, C75X, L76X, S77X, D78X, A79X, G80X, H81X, E82X, H83X, D84X, T85X, W86X, F87X, D88X, T89X, M90X, L91X, G92X, F93X, A94X, I95X, S96X, A97X, Y98X, A99X, L100X, K101X, S102X, R103X, I104X, A105X, L106X, T107X, V108X, E109X, D110X, S111X, P112X, Y113X, P114X, G115X, T116X, P117X, G118X, D119X, L120X, L121X, E122X, L123X, Q124X, I125X, C126X, P127X, L128X, N129X, G130X, Y131X, C132X, E133X, wherein the first letter is the native amino acid appearing at the position of the indicated number, and the last letter is the amino acid substituted for the native amino acid. The "X" represents the deletion or replacement of any amino acid residue that, when introduced in place of the wild type amino acid residue, results in a mutant or variant pertussis toxin S5 subunit with altered characteristics for covalent modification or conjugation and, when coupled to bioactive compounds at that site, retains its ganglioside (sialoglycoproteins) binding activity.

Lysine Substitutions in the rCTB, rLTB, Shiga, Shiga-like, and pertussis family

Conjugation of members of the rCTB, rLTB, Shiga, Shiga-like, and pertussis family to immunogens derived from infectious diseases can create vaccines which induce protection following mucosal delivery. Classic conjugation chemistry targets are reactive amino acid side chain groups such as primary amines, carboxylic acids, aldehydes, or sulfhydryls. The lysine residues are distributed at various places throughout the sequence. Those lysine residues that are near the Gm-1 binding site can couple to the ligand in a conformation which interferes with ganglioside binding. Accordingly, the invention articulates a number of mutant conjugation characteristics, primarily at lysine residues found in the rCTB protein family.

Therefore, the present approach involves the idea of directing the covalent linkage of carrier and immunogen away from the ganglioside receptor of CTB family. Site directed mutagenesis is employed to create a panel of mutations at various lysines of the rCTB family

coding sequences. These mutations will result in mutants which produce variable levels of protein, *in vitro*, comparable to those of the wild type and which can effectively deliver bioactive CTB-immunogen complexes to the mucosal immune system.

Accordingly, the invention articulates a number of mutant rCTB family proteins that with amino acid substitutions that replace the lysine residues found in the wild type rCTB family protein. Preferably, the following amino acid substitutions are contemplated for rCTB family proteins GI:758351, 808900, 2144685, and 48890 (wherein "X" is any amino acid that results in a rCTB protein with a reduced ability to conjugate at the mutant sites): K44X, K55X, K64X, K83X, K84X, K90X, K102X, K105X, and K112X. For rCTB family proteins GI:1827850, 998409, and 1421511, preferably K34X, K43X, K62X, K63X, K69X, K81X, K84X, and K91X. For rCTB family proteins GI: 229616, preferably K43X, K63X, K69X, K81, K84X, and K91X. For rCTB family protein GI: 2781121, preferably K35X, K44X, K62X, K69X, K81X, K84X, and K91X. For rCTB family protein GI:1421525, preferably K43X, K62X, K63X, K69X, K81X, K91X. For all rCTB family B subunit proteins if they have a lysine at position 91, most preferably K91X.

For LTB and the LTB family proteins GI:3062900, 1169505, 1395122, 145833, 1648865, 223254, 408996, and 69630 wherein "X" is any amino acid that results in a protein with a reduced ability to conjugate at the mutant sites, K44X, K55X, K64X, K83X, K84X, K90X, K102X, and K105X. For LTB family protein 494265 preferably K34X, K43X, K62X, K63X, K69X, K81X, K84X, and K91X. For all LTB family proteins if they have a lysine at position 91, most preferably K91X

For LT-IIa protein GI:146671 wherein "X" is any amino acid that results in a protein with a reduced ability to conjugate at the mutant sites, K47X, K48X, K89X, K100X, and K107X.

For LT-IIb protein GI:576584 wherein "X" is any amino acid that results in a protein with a reduced ability to conjugate at the mutant sites, K48X, K76X, and K89X.

For Shiga toxin beta subunit protein GI:152784 wherein "X" is any amino acid that results in a protein with a reduced ability to conjugate at the mutant sites, K43X, K47X, and K73X.

For Shiga-like toxin from *E.coli* subunit B protein GI:4877349 wherein "X" is any amino acid that results in a protein with a reduced ability to conjugate at the mutant sites, K41X, K45X, and K71X.

For pertussis toxin protein GI:144070 wherein "X" is any amino acid that results in a protein with a reduced ability to conjugate at the mutant sites subunit S2: K98X, K167X, K177X, K195X. S3: K38X, K100X, K121X, K133X, and K196X. S4: K50X, K61X, K78X, K96X, K99X, K127X, K134X, and K148X. S5: K42X, K52X, K54X, K56X, and K101X.

Lysine additions in rCTB family subunits

The invention further contemplates the addition of amino acid residues throughout the amino acid sequence of the rCTB family, the LTB family, Shiga toxin, Shiga-like toxin, and pertussis toxin to direct conjugation of various bioactive compounds such as immunogens, immunomodulators, or drugs to specified sites. Any residue can be mutated to introduce amino acid residues that promotes acceptable conjugation events.

Hydrophobic coupling of rCTB

Although chemical coupling is an excellent way to produce Gm-1 targeted immunogens, it would be highly advantageous to alter rCTB in such a way that it no longer requires chemical coupling, but instead strongly binds to other proteins or immunogens non-covalently. As an example, if rCTB could be prenylated it would then display up to 5 hydrophobic isoprene tails per pentamer. It is expected that in the properly folded and assembled protein, these tails would be present on the surface of the protein in opposite orientation to the Gm-1 binding surfaces. The commonly occurring isoprene groups include farnesyl, geranyl, geranyl-geranyl; all other prenylation or hydrophobic modification are considered to achieve the same outcome. Cholera toxin thus modified is expected to have an affinity for hydrophobic structures and surfaces. Prenylated rCTB would bind to bacterial cell walls, cell membranes, virus membrane coats, and other hydrophobic structures such as hydrophobic proteins and hydrophobic phospholipids or adjuvants. The isoprene groups on rCTB would spontaneously insert into any lipid membrane, or hydrophobic miscelle, to non-covalently associate rCTB with the hydrophobic structure in such a manner as to present the ligand binding site of rCTB on the outer surface of the micelle. This concept could be applied to making conjugates between rCTB and vaccine antigens or to promote ganglioside specificity in microsome or liposome delivery systems.

Prenylation naturally occurs on selected proteins in eukaryotic cells, such as GTP binding proteins. The post-translational modification of proteins occurs at the C-Terminus, with a consensus sequence of CAAX (SEQ ID NO: 34) where C=cysteine, A=aliphatic amino acid, and X=any amino acid. The isoprenyl group is attached to the cysteine via a thioether link and after modification the cysteine becomes the last amino acid in the sequence. Other post-translational modifications such as modification of proteins with lipids, fatty acids, and glycosyl phospholipids also occur and could potentially serve the same purpose as prenylation.

In one embodiment, the gene for cholera toxin B is modified to include a prenylation signal that would permit *in vitro* prenylation. This may include a C-terminal cysteine, C-terminal CAAX box sequence (SEQ ID NO: 34), or any other sequence necessary or sufficient to achieve *in vitro* or *in vivo* prenylation. A plasmid containing the mutant cholera toxin B gene is transfected into a suitable host for expression. For example, a high expression rCTB vector is used to express

the mutated rCTB in bacteria. Once the mutated rCTB has been expressed and purified, it is used as a substrate for *in vitro* prenylation. *In vitro* prenylation is performed with the appropriate prenylation enzymes and substrates. The prenylated rCTB product is purified as necessary and used to produce the desired conjugated product. Alternatively, if the prenylation reactions permit, prenylation of rCTB is performed in the presence of the conjugation partner thus allowing immediate binding of the modified rCTB to the immunogen or vaccine. This may serve to stabilize prenylated rCTB or facilitate production and purification.

An alternative embodiment involves co-transfecting bacterial or eukaryotic cells to produce rCTB and the necessary proteins for prenylation. In this embodiment it may be necessary to construct the proteins for the special requirements of each system. For example, in any expression system both the rCTB and prenylation proteins may need to be targeted to the same compartment for efficient prenylation. This could be the cytoplasm, periplasm, or extracellular milieu of bacterial, or the cytoplasm, any of the secretory compartments or other organelles in eukaryotic expression systems.

Confirmation of hydrophobic coupling, purification, and Gm-1 binding assays would be performed essentially the same as chemically coupled rCTB, with variations in buffer and pH. Vaccination would also be performed in essentially the same way.

Vaccines

These concepts have been contemplated as methods for producing cholera toxin B based vaccines. In particular, these prenyl-rCTB constructs lend themselves to whole cell, whole virus, or hydrophobic particle vaccine conjugates. Vaccines contemplated include influenza virus vaccines, both whole and split, because influenza virus acquires a membrane coat and is thus capable of binding prenyl-rCTB to the membrane coat. The split virus vaccines commonly contain the membrane coat protein hemagglutinin. This protein binds to prenyl-rCTB through hydrophobic patches on its surface. Another suitable vaccine is whole cell pertussis, where prenyl-CTB could insert the hydrophobic groups into the outer membrane of B. pertussis. Also, hydrophobic particles such as water in oil emulsion based vaccines and liposome based vaccines would be attractive candidates for this technology.

Genetic fusion of rCTB with an immunogen

Another option for production of an rCTB coupled immunogen is genetic fusion. Genetic fusion would occur using a vector that contains a promoter for expressing the fusion protein, the DNA sequence of the cholera toxin binding subunit rCTB, and an immunogenic peptide coding sequence. The rCTB and the immunogenic peptide coding sequence would be linked such that they were in the proper reading frame producing a fusion protein. The fusion protein would be

expressed, secreted, and purified for use as a vaccine. rCTB fusion proteins are as shown in Table 2.

TABLE 2: CTB FUSION PROTEINS

Plasmid	Bacterial host	Product Form	Expression level (μg/ml)	Protein
λ17 GST 1.10*	XL-1	Inclusion bodies	96	NH2-GST-linker #1-A8VD4-C
λ17 GST2.9**	XL-1	Inclusion bodies	58	NH2-GST-linker #2-A8VD4-CT
P55-64gp12***	*V. cholera*	Secreted	110	CTB with internal HIV epitope

* Other antigenic sequences may be substituted for A8VD4. Linker is GAPAS (SEQ ID NO 34).

** Other antigenic sequences may be substituted for A8VD4. Linker is PGSTRAADRDGAPAS (SEQ ID NO: 35).

*** HIV epitope is flanked by KpnI and MscI restriction enzyme sites, allowing easy insertion of other coding sequences into CTB.

rCTB vaccines

Microorganisms which invade via mucosal membrane surfaces are the best targets for rCTB vaccines. However, it could be used for almost any type of vaccine. Examples of diseases and antigens that could be coupled to rCTB for production of a vaccine are listed below in Table 3. As mentioned above, larger, whole and killed cell vaccines or whole virus vaccines could be produced particularly using the hydrophobic coupling. Vaccines which couple the rCTB protein in some way to an immunogen of choice would be particularly useful for any type of disease which is targeted to mucosal surfaces.

Production of a specific vaccine involves the steps of 1) identifying an antigen or immunogen; 2) coupling the antigen or immunogen to rCTB in some way (chemically, hydrophobically, or genetically); 3) isolating the coupled rCTB/immunogen and confirming that it still binds to Gm-1; 4) identifying that it works *in vivo* by injecting it into a mouse or other model animal; and 5) testing it for efficacy in humans or other primates. Examples of vaccine targets are shown in Table 3 with potential antigens.

TABLE 3: HUMAN INFECTIOUS DISEASES AND VACCINE ANTIGENS

Oral/Intestinal Diseases	Infectious agent	Exemplary Antigen
	H. pylori	Urease, 50 kDa citrate synthatase homologue protein, bacterial lysates, whole organisms
	E. coli	LPS, enterotoxin type I, II, whole organisms
	V. cholera	Killed bacteria, avirulent live
	Rotavirus	VP2, VP4, VP6, VP7, whole viral particles, VLPs
	Salmonella typhi	Whole or fragments of organism
	Shigella dysenteria	Whole or fragments of organism
Respiratory Diseases		
	H. influenza	Current vaccines are conjugates
	RSV	F/G protein,whole viral particles, VLPs
	Corynebacteria diphtheria	Toxoid, whole or fragments of organism
	Bordetella pertussis (Whooping cough)	Toxoid, filamentous hemagglutinin, pilus, pertactin, S1 subunits serotypes, whole or fragments of organism
	Streptococcus pneumonia	Polysaccharide ags (23) 23 most common serotypes of 90 capsular serotypes currently targeted. New vaccines target 7-11 serotypes bound to a protein carrier, whole or fragments of organism
	Influenza virus	Killed, lysates, HA(hemagglutinin), NA (neuraminidase), trivalent or more based on most common serotypes that year; whole virus
	Anthrax	Whole or fragments of organism
Urogenital diseases (STD's)		
	HIV	Gp41, Gp120 env, Gp160env, Tat protein, multiepitope polypeptide TAB9, Gag, Pol, and Env proteins
	Chlamydia	Whole or fragments of organism
	Treponema pallidum (Syphilis)	47 kDa antigen, whole or fragments of organism
	Neisseria gonorrhea	whole or fragments of organism
	Herpes	whole viral particles, VLPs, Tk

Use of rCTB in treatment of autoimmunity and the induction of tolerance

Autoantigen specific peripheral T cell tolerance is induced by CTB-autoantigen conjugates. Peripheral tolerance suppresses autoinflammatory disease by down regulating Th1. CTB-autoantigens can even reverse existing inflammatory cell proliferation in animals. Autoimmune diseases considered for treatment using CTB include without limitation: rheumatoid arthritis, encephalomyelitis (or other neuron demyelinating diseases), diabetes, and female anti-paternal immuno-contraception.

Reversal of inflammatory cell proliferation (th1 tolerization) was shown in autoimmune models for diabetes in NOD mice (insulin-CTB conjugates; Bergerot et al. *PNAS* 1997 94:4610-14.). Additionally it was shown for rheumatoid arthritis (HSP 60, Haque et al. 1996 *EurJImmunol* 26:2650-2656; collagen type II conjugates) and experimental autoimmune encephalomyelitis (EAE) in Lewis rats (Sun et al., Myelin basic protein MBP-peptide CTB conjugate, 1996 *PNAS* 93:7196-7201). These conjugates were fed as single doses to animals before and after induction of disease.

It is contemplated that the rCTB of the invention can be chemically, hydrophobically, or genetically coupled to the above-mentioned antigens and fed to animal models to test the efficacy. It is believed that because such a treatment was effective in the above examples it will be as effective or more so using the advantageous rCTB of the invention. Additionally, the rCTB conjugates of the invention are easier to make, more likely to work because the site of conjugation can be controlled, and yield a higher percentage of conjugated products that in turn will induce both a systemic and mucosal response.

The rCTB or other B subunits of the invention can also be used to induce tolerance to infection. For example, tolerization may be induced by administering the *Leshmania major* produced immunodominant antigen LACK, which generates a $CD4^+$ T cell dominant response and drives a th2 response to associated antigens. Th2 facilitates lesion development and leads to unresolved infection. LACK+ transgenic mice are tolerant of LACK and resolve infection. LACK fed animals are tolerized and resolve infection. (McSorley et al, *Eur.J.Immunol.* 1998; 28:424-32.) showed that CTB-LACK, fed or given nasally, down regulated the T cell proliferative response to LACK and allowed animals to resolve (fight off) disease more effectively. Additionally, responses to diseases associated with the development of acute autoimmunity such as Reiter's syndrome or Lyme disease can be treated with tolerizing conjugates.

EXAMPLES

EXAMPLE 1

Expression and Purification of Mutant rCTB

Production of Recombinant CTB in Bacteria

The expression plasmid MS-0 (see Figure 2) was used to express rCTB and variants. MS-0 containing the rCTB gene is named pML-CTBtac1. The plasmid pML-CTBtac1 surprisingly generates up to five times the product which was generated by a comparable plasmid (SBL's vector pJS162). PML-CTBtac1 was constructed by cloning a portion of the CTB genomic region and the complete CTB coding region into plasmid MS-0 creating a 3.66 Kb expression plasmid. The PvuII

site in the polylinker was destroyed during cloning. The plasmid contains a tac promoter from pKK223, an EcoRI-BamHI polylinker fragment, and can be found at genbank accession No M77749. The encoded protein is identical to the sequence from *V. cholera* strain 569B (SEQ ID NO: 2). The signal sequence (SEQ ID NO: 3) is also from the CTB *V. cholera* classic strain 569B CTB gene. The complete nucleotide sequence of *V. cholera* strain 569B CTB gene is shown in Figure 1 (SEQ ID NO:1). The signal sequence for LTB (SEQ ID NO:15) is MNKVKCYVLFTALLSSLCAYG and can be used in the production of mutants or variants of LTB.

Measurement of Expression Level of rCTB mutant or variant

It is advantageous to purify large quantities of the immunogen of interest quickly, easily and inexpensively. Accordingly, the ability to isolate milligram quantities of rCTB and rCTB variants for use in production of vaccines is discussed below.

A protocol for the isolation of rCTB and rCTB variants from shaker flasks is presented. Surprisingly, is was possible to obtain up to 800 μg of rCTB per ml of culture supernatant. Many recombinant expression systems are considered very productive with yields exceeding 10 μg/ml. The expression level of rCTB mutants thus far produced are moderately lower than wild type.

The concentration of all proteins tested is measured by comparison to a standard curve of rCTB at defined concentrations in the Gm-1 ELISA. The values recorded for a serial dilution of the unknown sample are then used to determine the sample concentration which is then multiplied by the dilution factor to obtain the correct concentration of the unknown sample.

One limitation of the Gm-1 ELISA is that it only identifies the relative binding concentrations of wild type vs. mutant rCTB. Thus, actual concentrations must be confirmed by Å280 or by Lowry, Bradford, BCA or similar colorimetric protein assays known by those practiced in the art.

Any mutation to the rCTB sequence might result in an increase or decrease in receptor binding affinity for Gm-1 and other ligands (Backstrom et al. 1997 *Mol Microbiol* 24:489-497; Merritt et al. 1995 *Structure* 3:561-570). Any significant change in receptor affinity of a newly created mutant rCTB for Gm-1 may cause the loss of binding in a Gm-1 ELISA and result an "apparent" decrease in concentration. In such case, each test protein is initially purified to homogeneity using methods discussed below. From these, the purity is demonstrated by comassie or silver stained polyacrylamide gel electrophoresis, a technique well known in the art. The relative concentration of each protein is determined by Å280 and confirmed by protein concentration assays. Equivalent concentrations of each protein, sufficient to saturate the ganglioside moiety (e.g. ~50 μg/ml), are incubated in a Gm-1 ELISA plate. A two-fold serial dilution of each mutant or unlabeled wild type protein is then incubated in solution with a constant concentration of horse radish peroxidase labeled wild type CTB (at concentrations which alone

generate a maximum signal, usually >~10 ug/ml). From this assay, a standard curve for each mutant can be generated and compared to a similar curve for unlabeled wild type (positive standard curve control). Discrepancies from the positive standard curve control reflect significant changes in the affinity of the mutant rCTB for gm-1. The difference between curves can be quantified to determine the quantitative differences in affinity assuming the wild type affinity of 4.6×10^{-12}M.

Purification of rCTB and rCTB variants

A number of CTB purification protocols are available and known in the art. Three purification methods are discussed below.

Ammonium Sulphate Precipitation of rCTB

This procedure worked well for cultures grown in baffled shaker flasks containing 100-500 µg/ml rCTB in the supernatant. Culture volumes between 50-250 ml have been successfully processed using this protocol. The supernatant was processed immediately after growth and clarification of the culture.

Briefly, the bacteria expressing rCTB or mutants of rCTB were grown in a shaker flask. The supernatant was clarified by centrifuging the cells at 7000 x *g* (avg.) for 15 min at 4°C into a pellet. A step wise precipitation of protein with increasing concentrations of ammonium sultate results in highly purified rCTB product. Accordingly, ammonium sulfate was gradually added to the culture supernatant to bring the ammonium sulfate concentration up to 30% (vol/vol) while stirring at room temperature. After 1 hour of precipitation without stirring, the sample was centrifuged at 14,000 x *g* (avg.) for 15 minutes at 4°C. The supernatant was removed and a concentrated ammonium sulfate solution was added to bring the concentration to 50% (vol/vol) while stirring. This mixture was allowed to stand at room temperature for 1 hour. The sample was again centrifuged at 14,000 x *g* (avg.) for 15 min at 4°C. A third precipitation step was performed, bringing the concentration up to 65% (101 g/L total volume). This mixture was again stirred and allowed to stand for 1 hour before centrifugation as before. The pellet of highly purified rCTB was then resuspended in a suitable buffer, for example, if additional purification by ion exchange chromatography is desired, the sample was brought up in 10 mM $NaPO_4$ buffer. The sample was then placed in dialysis tubing (7000 MWCO). If the sample is not immediately used, it can be stored undialyzed for up to one month at 4°C until ready to use. When dialysis was performed, one-liter volumes of buffer were used for at least one hour each, changing the buffer three times.

Purification of rCTB by DEAE Ion-exchange Chromatography

This part of the protocol applies specifically to ammonium sulfate precipitated rCTB. A DEAE Sepharose Fast Flow gel of choice was packed on an Amersham Pharmacia Biotech column following the manufacturer's directions. 15 ml of a well-mixed gel slurry produced about 10 ml bed volume. The flow rate for packing was at least 4 ml/min. A column size of 1.5 cm (inner

diameter) and 20 cm (length) with a bed volume of 10 ml bound approximately 30 mg of rCTB from culture processed by ammonium sulfate precipitate. A flow rate adapter was used with the column. The column was washed with 1 M NaCl to activate the column bed, or 2 M NaCl to regenerate a spent column bed. The flow rate for all washes did not exceed 75% of the packing rate, or 3 ml/min. The column was extensively equilibrated with Buffer A (10 mM Sodium Phosphate Buffer, pH 7.5) to remove all salt. The sample containing rCTB was applied at a rate of 1-1.5 ml/min. Each fractions was tested for the presence of rCTB. Following sample application, the column was washed with Buffer A at a flow rate of 3 ml/min until the optical density at Å280 dropped below 0.02, confirming the removal of all non-specific and cationic species. The column was washed with Buffer A containing 50 mM NaCl, until the optical density at Å280 dropped below 0.02. RCTB was eluted from the column with Buffer A containing 100 mM NaCl. All fractions were collected and pooled. Approximately 80% of the total column bound rCTB was recovered in these fractions. The column was washed with Buffer A containing 1 M NaCl until the optical density at Å280 dropped below 0.02. Before regeneration of the column, the DEAE bed was washed with 10 mM sodium phosphate, pH 7.5 to remove all of the salt. Alternatively, CTB bound to the DEAE column can be eluted with a salt gradient from 0.02M NaCl to 1 M NaCl in buffer A. Fractions containing rCTB are pooled, dialyzed or concentrated in the buffer of choice.

Detailed protocol for purification of rCTB using affinity chromatography on a ganglioside-Affigel column

In this technique, clarified bacterial culture supernatant was directly poured onto the column. rCTB or rCTB mutants bound specifically to the immobilized gangliosides and the column was washed. rCTB was then eluted with 5M guanidine thiocyanite and dialyzed against a phosphate buffer. This technique was very easy and a high purity was achieved. However, there was a limited capacity of approximately 7 mg rCTB per run and it required denatured protein to be able to fold properly, which may be a problem with some mutants.

The Ganglioside-Affigel columns were produced using Affigel (Pierce, Rockford, IL). The smaller Ganglioside-Affigel column (0.5 ml bed volume) had the capacity to bind 1-2 mg of rCTB, while the larger (5 ml bed volume) will bind at least 7 mg. The following buffers were used: 0.1 M Na-phosphate buffer, pH 7.5; 0.1 M Na-phosphate buffer, pH 7.5, 0.5 M Na Cl; 5 M guanidine thiocyanite in DI water, freshly made in a 50 ml conical tube.

The culture supernatant was centrifuged at 10,000 rpm for 10 min (SS-34). Particulate matter was avoided as it clogs the column. The column was equilibrated with 0.1 M Na-phosphate buffer, pH (2-5 column volumes). The sample containing rCTB was added and the flow through was collected and applied once more to allow quantitative binding to the column. The column was washed with 0.1 M Na-phosphate buffer, pH 7.5 until the Å280 was close to 0, the column was

washed again with 0.1 M Na-phosphate buffer, pH 7.5, 0.5 M NaCl until the Å280 was close to 0. The column was equilibrated with 0.1 M Na-phosphate buffer, pH 7.5 (2 column volumes). Fresh guanidine thiocyanite solution was prepared in 1 ml steps, 1 ml fractions were collected. The column was allowed to sit for 10 min after addition of the first two 1 ml additions of the elution buffer to the column. This ensured complete elution of rCTB from the ganglioside resin. The Å280 containing fractions were pooled and dialyzed against 500 ml of 0.1 M Na-phosphate buffer, pH 7.4, with no stirring. A total of 4 buffer changes were performed with the last two in the desired buffer in which to store the protein.

Optionally, rCtB can be purified by cation exchange on a CM Sepharose Fast Flow ion exchange column. Briefly, NH_4SO_4 salt precipitated or DEAE eluted rCTB can be dialyzed against 20 mM Tris-HCl, 10 mM NaCl, pH 8.4 and the dialysate applied to the column.

After washing, a highly purified rCtB product can be eluted with a continuous salt gradient of 10 mM to 300 mM NaCl in 20 mM Tris-HCl, pH 8.4.

This method resolves the remaining few contaminant products we see following DEAE anion exchange chromatography.

EXAMPLE 2

Mutagenesis of rCTB

Mutagenesis was initially performed by oligonucleotide-directed *in vitro* mutagenesis according to Kunkel, TA, Roberts, J., and Zakour, R. "Rapid and efficient site-specific mutagenesis without phenotypic selection", *Methods Enzymol.*, 154, 367-382, 1987. Subsequently mutagenesis was performed essentially according to Nelson, M. and McClelland, M., Methods Enzymol. 216, 279-303, 1992. The results of the mutagenesis were confirmed by DNA sequencing.

EXAMPLE 3

Lysine Mutation of rCTB

The following mutant rCTB proteins are made using the methods described in Example 2. A lysine residue is inserted into the rCTB sequence of SEQ ID NO 2 to replace amino acid Asn 103. Asparagine's polarity comes from an amino side chain which is uncharged at neutral pH. Replacement of Asn 103 with a lysine may prove to be an effective and novel conjugation site. A lysine residue is inserted into the rCTB sequence of SEQ ID NO 2 to replace the amino-terminal amino acid Ala 102. A lysine residue is inserted at position Ala1. A lysine residue is inserted at position Pro2. Although this mutant changes the structure of the amino terminus, Gm-1 binding affinity and antigenicity of the resulting composition is not compromised by the mutation.

Amino acids 14-20 form a β-sheet on the outer surface of rCTB with many of the side chains facing outward. Of the amino acid residues that form this structure, His 18, is particularly suitable for mutagenesis. Accordingly, a mutant rCTB protein is generated having the mutation

H18K. Substitution of His 13 for any other amino acid may effect Zn^{+2} binding characterisitics of rCTB as well as pentamer-pentamer self association. Elimination of either of these characteristics might be desired for purposes of biochemical isolation of the mutant proteins.

EXAMPLE 4

Determining Immunogenicity of rCTB Mutants Coupled to Immunogens

Immunogenicity is determined by injection of the rCTB:Immunogen into balb/c mice. 1:1, 1:6, and 1:3 ratios of rCTB:immunogen conjugate are injected. Additionally, a control using immunogen alone is injected injected. After ten days, blood is drawn from the immunized mice and antibodies to the rCTB:immunogen conjugate are identified and measured via an antibody assaying protocol such as ELISA or any other suitable assay widely known by one of skill in the art.

EXAMPLE 5

Chemical Coupling of Mutant rCTB Proteins to an Immunogen

Lysine residues

Mutant CTB proteins CTB K91G and CTB-K62G, 63G, 91G were modified, expressed, and purified using the method described in Examples 1-2. Mutation of K91 was chosen as a site for substitution because of its proximity to the Gm-1 binding site of CTB and it known interaction with the terminal glactose of Gm-1. Further, as discussed above, K91 is highly reactive with small molecular conjugates, such as FITC or SPDP. Accordingly, conjugations with heterobifunctional reagents are likely to create molecular complexes with diminished Gm-1 binding affinities. some important modifications to protocols which create a simpler, more efficient coupling reaction with SPDP. In brief, the pH of the conjugation must be > 8.0, and the SPDP (N-Succinimidyl 3-[2-pyridyldithio]propionate) modified rCTB and its associated molecule must be highly concentrated.

Employing these modifications, rCTB wild type was successfully coupled to bovine serum albumin (BSA) and to Diphtheria toxoid (Dt).

In contrast to established protocols, neither BSA nor Dt was SPDP modified prior to conjugation. These proteins were determined to have free sulfhydryl groups which will readily react with the SPDP modified rCTB.

Modification of rCTB using SPDP chemistry

rCTB or variant rCTB was dialyzed into Borate-Phosphate buffer. Borate-Phosphate buffers: a 10X stock solution (2L) was made by adding 12.4 g. Boric acid, 175.3 g NaCl, and 136.09 g Potassium phosphate (monobasic) into 1.5 liters of water. The solution was adjusted to a pH of 6.6 with 5M KOH and then brought up to a final volume of 2 liters with water. The solution was filtered through 0.2 micron filter. The pH of a 1X solution was 7.0. Borate-Phosphate Buffer, pH 8.5 was 1X buffer adjusted with 5M KOH. The rCTB was modified using ten-fold molar excess of SPDP over rCTB. The SPDP was weighed and diluted to 5 mg/ml using anhydrous

N,N-dimethylformamide (DMF), containing the molecular sieves (Grade 562, Type 3a, 4-8 mesh beads, Fisher Scientific, Tustin, CA). Thus, 25 µl of 5 mg/ml SPDP would be added to a 2 mg rCTB/ml solution of rCTB. The SPDP and rCTB were incubated and allowed to react for at least 1 hour at room temperature.

Excess SPDP was removed by running a G-25 gel filtration column (1.5 cm x 9 cm bed volume is the minimum required to separate excess SPDP). 10 mM sodium phosphate buffer, pH 7.5 containing 50 mM NaCl was used for column running buffer. The SPDP modified rCTB was concentrated by using Amicon centricon c-30 (30 kD cut-off) to a volume between 1-2 ml.

Conjugation of protein to SPDP modified rCTB

Unmodified protein (the immunogen - i.e. BSA or Dt) was dialyzed against 3 x 1000 volumes Borate-Phosphate buffer, pH 7.0. SPDP modified rCTB and unmodified protein (immunogen) were combined at selected molar ratios and incubated overnight. The next the sample was concentrated using a Centricon 30 (30,000 MWCO Millipore, Bedford, MA).

Confirmation of Chemical Coupling

The concentrated sample was separated into its component parts by gel electrophoresis on a 4-20% Tris-Glycine gel to confirm the formation of conjugates. A number of techniques can be used to confirm chemical coupling of the two proteins including: Gm-1 ELISA using immunogen-specific antibodies which recognize only immunogen when bound to rCTB, and molecular seive gel filtration.

Cysteine residues

Mutant CTB proteins CTB-H18C and CTB-M101C were modified, expressed, and purified using the methods described in Examples 1-2. The rCTB proteins were treated with 100 mM DTT for 2 hours at room temperature. DTT was removed by centrifugation for 2 min at 2000g through a 1 ml spin column packed with Biogel P6 polyacrylamide gel (BioRad, Cambridge, MA), equilibrated with 25 mM sodium phosphate buffer pH 7.0/10 mM ethylenediaminetetraacetic acid (EDTA). The collected fraction was incubated for 2 hours at room temperature with maleimide-activated immunogen (i.e. immunogen functionalized with the cross-linker SMCC, Sigma). The reaction was stopped by the addition of β-mercaptoethanol (β-me) to a final concentration of 1.5 mM and N-ethylmaleimide (NEM) suspended in N,N-dimethylformamide to a final concentration of 3 mM.

Confirmation of Chemical Coupling

Conjugation of immunogen to rCTB was assessed by ELISA and Western blotting. (I) ELISA: conjugates of Immunogen and rCTB were incubated with GLM1-coated microtitre plates and immobilized Immunogen was detected with the chromogenic substrate 3,3',5,5' tetramethylbenzidine (TMB, KPL). Detection of plate bound immunogen which was dependent on a coating of GM-1 was interpreted as evidence that rCTB was physically linked to the Immunogen. (II) Western Blots:

conjugates were separated by electrophoresis on 7.5% SDS polyacrylamide gels and transferred to nitrocellulose. rCTB was detected with polyclonal anti-rCTB sera, followed by goat anti-mouse Ig-alkaline phosphatase (A)(Amersham, Buckinghamshire, UK) and AP-specific substrate (BCIP/NBT, BioRad, Cambridge, MA). Immunogen was detected with antibodies specific to the immunogen, goat anti-mouse Ig-AP and BCIP/NBT.

Purification of immunogen-rCTB Conjugates

Conjugates were purified by size exclusion chromatography based on the expected size of the conjugate. Additional purification was performed within a GM-1 or ganglioside affinity column to eliminate nonconjugated immunogen and within an antigen or immunogen specific column to eliminate residual free rCTB and rCTB-rCTB conjugates, respectively. This left only the bioactive conjugates which were assessed by SDS-PAGE and ELISA as described in Example 6.

EXAMPLE 6

Assaying Mutant rCTB Conjugated Complexes for Gm-1 Binding Relative to Wild Type rCTB

Maintenance of the ganglioside affinity by the GM-1 binding sites in mutant CTB, wild type CTB, or conjugates derived therefrom can be assayed by ELISA analysis. In the first example, the monosialoganglioside, GM-1, was used at a concentration of 0.3 nmol/ml PBS. One hundred microliters of ganglioside solution was added to each well of a 96 well microtiter plate and incubated overnight at 22°C. The following day, the plate was washed twice with PBS utilizing a Bioplate Autowasher EL-404 (Bio-Tec Instruments, Winooski, VT). All wells of the plate were then coated with 200 µl of a 0.1% BSA in PBS solution for 30 minutes at 37°C. The plate was washed three times in PBS containing 0.05% tween-20 and evacuated of remaining solution. All wells, other than the initial test wells, received 100 µl of a solution of 0.1% BSA, 0.05% tween-20 in PBS. Test wells then received 150 µl of 15 µg CTB/ml test mutant, wild type, or conjugate. A three-fold serial dilution was then conducted across the plate, sequentially transferring and diluting 50 µl from the second test well into 100 µl buffer in the third well and so on for up to twelve dilutions. The plate was incubated for 90 minutes at 22°C. The plate was then washed three times as described above. All wells then received either 100 µl of a 1/20,000 dilution of mouse anti-rCTB antiserum or a pre-determined dilution of mouse anti-serum to the coupled compound. Wells were incubated one hour at 22°C and subsequently washed three times as above. Test wells then received 100 µl of a 1/3000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG and were allowed to incubate one hour at 22°C. Plates were then washed again and incubated for 20 minutes with 100 µl of a 1 mg/ml solution of othophenylenediamine dihydrochloride (OPD) and 0.01% H_2O_2 in 0.1 M sodium citrate buffer, pH 4.5. 50 µl of a 3N HCl solution was then added to stop the enzymatic reaction. Plates were then read at 492 nm and

650 nm in a SpectroMax 250 microplate reader (Molecular Devices, Inc., Sunnyvale, CA). Results for mutants or conjugates thereof were reported as a function of endpoint dilution (apparent concentration) relative to wild type or conjugates thereof, respectively. The results indicated that mutated rCTB conjugated complexes retained their ability to bind to GM-1 binding sites.

WHAT IS CLAIMED IS:

1. A recombinant AB_5 B subunit protein comprising at least one mutation, wherein said mutation alters the number of amino acid residues available for chemical modification as compared to a wild type AB_5 B subunit protein, and wherein said recombinant protein retains an effective target ligand binding affinity.

2. The recombinant AB_5 B subunit protein of Claim 1, wherein said mutation increases the number of amino acid residues available for chemical modification.

3. The recombinant AB_5 B subunit of Claim 1, wherein said mutation decreases the number of amino acid residues available for chemical modification.

4. The recombinant AB_5 B subunit of Claim 1, wherein said amino acid residues available for chemical modification are selected from the group consisting of lysine, cysteine, and histidine.

5. The recombinant AB_5 B subunit of Claim 1, wherein said mutation is selected from the group consisting of an amino acid insertion, an amino acid deletion, and an amino acid substitution.

6. The recombinant AB_5 B subunit protein of Claim 1, wherein said recombinant AB_5 B subunit protein is in chemical association with a compound selected from the group consisting of drugs, immunogens, immunomodulatory molecules, adjuvants, and other bioactive molecules.

7. The recombinant AB_5 B subunit protein of Claim 1, wherein said AB_5 B subunit protein is selected from the group consisting of cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, and pertussis toxin B protein.

8. The recombinant AB_5 B subunit protein of Claim 1, wherein said AB_5 B subunit protein is cholera toxin B (CTB) subunit protein.

9. The recombinant cholera toxin B (CTB) subunit protein of Claim 8, wherein said recombinant protein retains an effective Gm-1 binding affinity.

10. The recombinant cholera toxin B subunit protein of Claim 8, wherein said mutation is selected from the group consisting of T1X, P2X, Q3X, N4X, I5X, T6X, D7X, L8X, C9X, A10X, E11X, Y12X, H13X, N14X, T15X, Q16X, I17X, H18X, T19X, L20X, N21X, D22X, K23X, I24X, F25X, S26X, Y27X, T28X, E29X, S30X, L31X, A32X, G33X, K34X, R35X, E36X, M37X, A38X, I39X, I40X, T41X, F42X, K43X, N44X, G45X, A46X, T47X, F48X, Q49X, V50X, E51X, V52X, P53X, G54X, S55X, Q56X, H57X, I58X, D59X, S60X, Q61X, K62X, K63X, A64X, I65X, E66X, R67X, M68X, K69X, D70X, T71X, L72X, R73X, I74X, A75X, Y76X, L77X, T78X, E79X, A80X, K81X, V82X, E83X, K84X, L85X, C86X, V87X, W88X, N89X, N90X, K91X,

T92X, P93X, H94X, A95X, I96X, A97X, A98X, I99X, S100X, M101X, A102X, N103X, wherein "X" represent any amino acid residue that alters the number of covalent modification sites within the recombinant CTB protein.

11. The recombinant cholera toxin B (CTB) subunit of Claim 10, wherein said amino acid residues available for chemical modification are selected from the group consisting of lysine, cysteine, and histidine.

12. The recombinant cholera toxin B (CTB) subunit of Claim 10, wherein said mutation is selected from the group consisting of an amino acid insertion, an amino acid deletion, and an amino acid substitution.

13. The recombinant cholera toxin B (CTB) subunit of Claim 10, wherein "X" represents an amino acid that decreases the number of amino acid residues available for chemical modification.

14. The recombinant cholera toxin B subunit protein of Claim 11, wherein said at least one mutation is selected from the group consisting of K91G, K91Q, K91S, K91P, and K91Y.

15. The recombinant cholera toxin B subunit protein of Claim 11, wherein said at least one mutation is selected from the group consisting of K34G, K35G, K62G, K63G, and K91G.

16. The recombinant cholera toxin B subunit protein of Claim 11, comprising the mutations K34G, and K91G.

17. The recombinant cholera toxin B subunit protein of Claim 11, comprising the mutations K62G, K63G, and K91G.

18. The recombinant cholera toxin B (CTB) subunit protein of Claim 10, wherein "X" represents an amino acid that increases the number of amino acid residues available for chemical modification.

19. The recombinant cholera toxin B subunit protein of Claim 18, wherein said protein comprises at least one mutation selected from the group consisting of A1K, P2K, Q3K, N4K, T6K, N7K, H18K, N21K, F25K, N44K, M101K, N103K, Q3C, N4C, T6C, N7C, H18C, N21C, F25C, N44C, M101C, and N103C.

20. The recombinant AB_5 B subunit protein of Claim 1, wherein said mutations comprises:

at least one modification decreasing mutation, resulting in said recombinant AB_5 B subunit protein having a decreased number of amino acid residues available for modification as compared to a wild type AB_5 B subunit protein; and

at least one modification increasing mutation, wherein said modification increasing mutation introduces an amino acid capable of chemical modification into a site

within said recombinant AB_5 B subunit protein previously incapable of chemical modification.

21. The recombinant AB_5 B subunit protein of Claim 20, wherein said AB_5 B subunit protein is selected from the group consisting of cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, and pertussis toxin B protein.

22. The recombinant AB_5 B subunit protein of Claim 20, wherein said AB_5 B subunit protein is cholera toxin B protein (CTB).

23. The recombinant AB_5 B subunit protein of Claim 20, wherein said amino acid residues available for chemical modification are selected from the group consisting of lysine, cysteine, and histidine.

24. A method of making a recombinant AB_5 B subunit gene encoding the recombinant AB_5 B subunit protein of Claim 1, comprising:

providing an AB_5 B subunit gene encoding an AB_5 B subunit protein;

selecting codons encoding amino acid residues involved in covalent modification of the AB_5 B subunit protein; and

mutating said codons such that the resulting amino acids are either incapable of covalent modification or possess enhanced modification capabilities.

25. The method of Claim 24, wherein said AB_5 B subunit protein is selected from the group consisting of cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, and pertussis toxin B protein.

26. The method of Claim 24, wherein said amino acid residues available for chemical modification are selected from the group consisting of lysine, cysteine, and histidine.

27. A method for producing the recombinant AB_5 B subunit protein of Claim 1 comprising the steps of:

obtaining a gene encoding the recombinant AB_5 B subunit;

adding to said gene a promoter, thereby producing an expression cassette;

introducing said expression cassette into a suitable host cell, and

cultivating said host cell under conditions whereby said expression cassette is translated into protein.

28. The method of Claim 27, wherein said AB_5 B subunit protein is selected from the group consisting of cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, and pertussis toxin B protein.

29. The method of Claim 27, wherein said AB_5 B subunit protein is cholera toxin B protein (CTB).

30. A gene construct for producing the recombinant AB_5 B subunit of Claim 1, comprising:

a promoter and a DNA sequence which encodes the recombinant AB_5 B subunit, operably linked in the proper reading frame.

31. The gene construct of Claim 30, wherein said AB_5 B subunit protein is selected from the group consisting of cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, and pertussis toxin B protein.

32. The gene construct of Claim 30, wherein said AB_5 B subunit protein is cholera toxin B protein (CTB).

33. A method for producing the recombinant AB_5 B subunit of Claim 1, comprising:

expressing the gene construct of Claim 30, in a suitable host cell; and recovering recombinant AB_5 B subunit.

34. A method of generating an immune response to a recombinant AB_5 B subunit, comprising:

providing the recombinant AB_5 B subunit protein of Claim 1 or Claim 20;

covalently modifying said protein with a dimeric cross-linking reagent with a first and a second functional groups, wherein said first functional group is in chemical association with the recombinant AB_5 B subunit protein of Claim 1 or Claim 20;

covalently modifying said second functional group with a compound; and

administering said modified protein to a host until said immune response is generated.

35. The method of Claim 34, wherein said immune response is an antibody response.

36. The method of Claim 34, wherein said AB_5 B subunit protein is selected from the group consisting of cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, and pertussis toxin B protein.

37. The method of Claim 34, wherein said AB_5 B subunit protein is cholera toxin B protein (CTB).

38. The method of Claim 34, wherein said immune response is a cellular immune response.

39. The method of Claim 34, wherein the covalently modified protein is mixed with an immunogen to produce a hydrophobically coupled protein prior to administering said protein to said host to generate said immune response.

40. The method of Claim 39 wherein said AB_5 toxin B protein is selected from the group consisting of: cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, pertussis toxin B protein.

41. The method of Claim 39 wherein said AB_5 toxin B subunit protein is cholera toxin B protein (CTB).

42. An expression vector comprising:

a promoter and a gene encoding for the AB_5 B subunit protein of Claim 1, wherein said AB_5 toxin B protein is selected from the group consisting of: cholera toxin B protein (CTB), *E. coli* heat labile toxin B protein (LTB), LT type IIa B protein, LT type IIb B protein, Shiga toxin B protein, Shiga-like toxin B protein, pertussis toxin B protein.

43. The expression vector of Claim 42, wherein said expression vector is a gene fusion vector, further comprising:

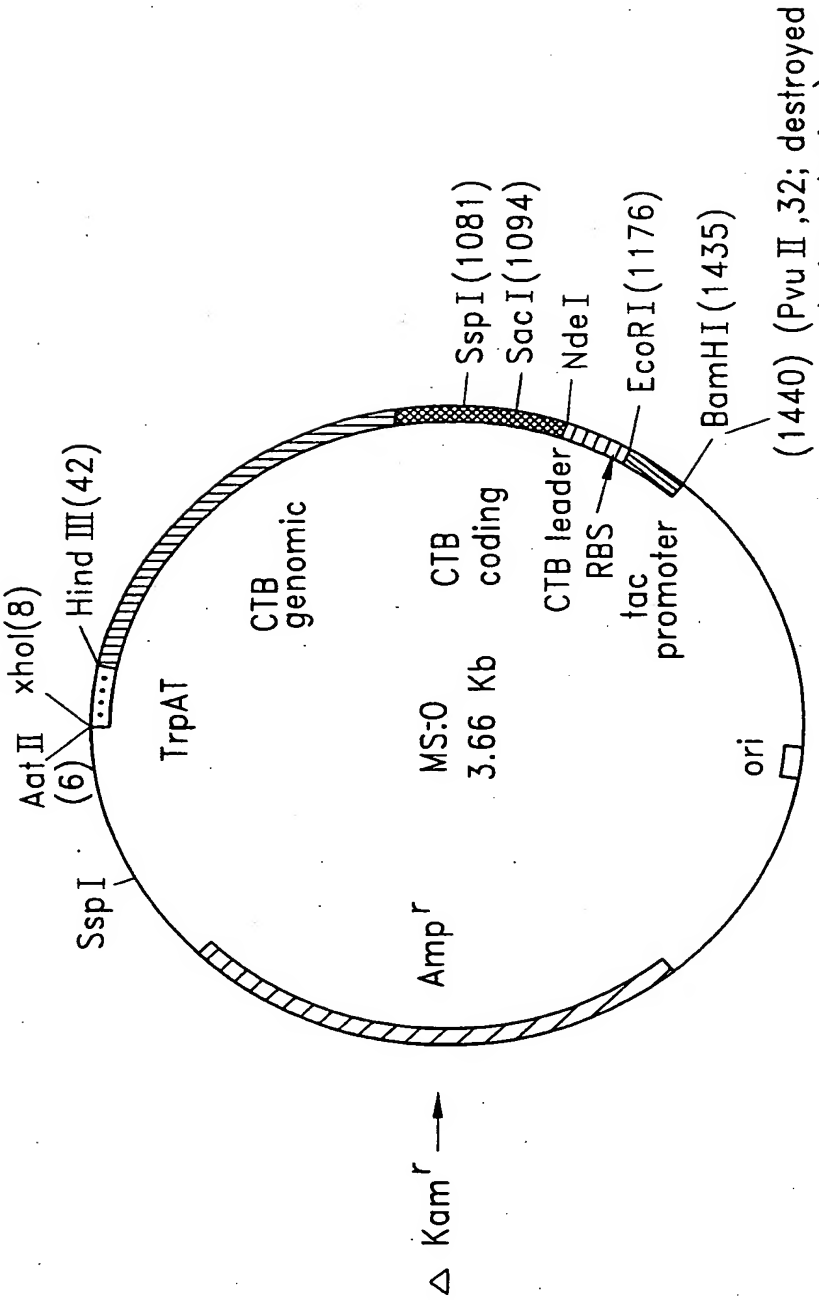
an immunogenic peptide coding sequence, wherein said AB_5 toxin B subunit protein and said immunogenic peptide coding sequences are operably linked in the proper reading frame, whereby a gene fusion protein results; and

said gene fusion protein is expressed and secreted.

44. The gene fusion vector of Claim 43, wherein said AB_5 toxin B subunit protein is cholera toxin B protein (CTB).

```
5'  ATG ATT AAA TTA AAA TTT GGT GTT TTT TTT ACA
    Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr

GTT TTA CTA TCT TCA GCA TAT GCA CAT GGA GCT CCT
Val Leu Leu Ser Ser Ala Tyr Ala His Gly Ala Pro

CAA AAT ATT ACT GAT TTG TGT GCA GAA TAC CAC AAC
Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn

ACA CAA ATA CAT ACG CTA AAT GAT AAG ATA TTT TCG
Thr Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser

TAT ACA GAA TCT CTA GCT GGA AAA AGA GAG ATG GCT
Tyr Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala

ATC ATT ACT TTT AAG AAT GGT GCA ACT TTT CAA GTA
Ile Ile Thr Phe Lys Asn Gly Ala Thr Phe Gln Val

GAA GTA CCA GGT AGT CAA CAT ATA GAT TCA CAA AAA
Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys

AAA GCG ATT GAA AGG ATG AAG ACC GAT CTG AGG ATT
Lys Ala Ile Glu Agr Met Lys Asp Thr Leu Arg Ile

GCA TAT CTT ACT GAA GCT AAA GTC GAA AAG TTA TGT
Ala Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys

GTA TGG AAT AAT AAA ACG CCT CAT GCG ATT GCC GCA
Val Trp Asn Asn Lys Thr Pro His Ala Ile Ala Ala

ATT AGT ATG GCA AAT T 3'
Ile Ser Met Ala Asn
```

FIG. 1



tac promoter is from pKK223; EcoR I – BamH I Fragment
Genbank Accession M77749

FIG. 2

Amino acid sequence (SEQ ID NO: 2), predicted secondary structure, position of lysine residues and regions involved in binding to GMI

```
                                10                                  20      23                  30
A P Q N I T D L C A E Y H N T Q I H T L N D K I F S Y T E S
                              GMI
CTTTTTTTTSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSTTTTTTTTTSSSSSSSSSHHH

        34                  40      43                  50                  60
L R G K R E M A I I T F K N G A T F Q V E V P G S Q H I D S
    GMI
TTTTTTTTHHHHSSSSSSSSSSTTTTTTTTTSSSSSSSSSSSSTTTTTTTTTTTTTTTT

  62 63                 69 70                          80 81       84              90
Q K K A I E R M K D T L R I A Y L T E A K V E K L C V W N N
GMI                                                             GMI
TTTTTTTHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHSSSSSSSSSSSSSTTTTTTT

91                  100
K T P H A I A A I S M A N
TTTTTTTTTTHHHHHHHHHHHHH
```

H=Helix; S=Sheet; T=Turn; C=Coil

- 9 lysine residues.
- 4 are at the top of CTB (23, 43, 81, 840).
- 4 are at the bottom of CTB, proximal to or in the GM1 binding site (34, 62, 63, 91).
- K69 is within the alpha helix.

FIG. 3

SEQUENCE LISTING

<110> ACTIVE BIOTECH AB
HANDLEY, Harold H.
HAAPARANTA, Tapio
EWALT, Karla L.
<120> AB5 TOXIN B SUBUNIT MUTANTS WITH ALTERED CHEMICAL CONJUGATION CHARACTERISTICS
<130> ACTBIO.004VPC
<160> 31
<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 373
<212> DNA
<213> Vibrio cholera
<220>
<221> CDS
<222> (1)...(373)
<223> The signal sequence from V. cholera classic strain 569B
<400> 1

```
atg att aaa tta aaa ttt ggt gtt ttt ttt aca gtt tta cta tct tca       48
Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser
 1               5                  10                  15
gca tat gca cat gga gct cct caa aat att act gat ttg tgt gca gaa       96
Ala Tyr Ala His Gly Ala Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu
            20                  25                  30
tac cac aac aca caa ata cat acg cta aat gat aag ata ttt tcg tat      144
Tyr His Asn Thr Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr
        35                  40                  45
aca gaa tct cta gct gga aaa aga gag atg gct atc att act ttt aag      192
Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys
    50                  55                  60
aat ggt gca act ttt caa gta gaa gta cca ggt agt caa cat ata gat      240
Asn Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
tca caa aaa aaa gcg att gaa agg atg aag acc gat ctg agg att gca      288
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Thr Asp Leu Arg Ile Ala
                85                  90                  95
tat ctt act gaa gct aaa gtc gaa aag tta tgt gta tgg aat aat aaa      336
Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
acg cct cat gcg att gcc gca att agt atg gca aat t                   373
Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
        115                 120
```

<210> 2
<211> 124
<212> PRT
<213> Vibrio cholera
<400> 2

```
Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser
 1               5                  10                  15
Ala Tyr Ala His Gly Ala Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu
            20                  25                  30
Tyr His Asn Thr Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr
        35                  40                  45
Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys
    50                  55                  60
```

```
Asn Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Thr Asp Leu Arg Ile Ala
                85                  90                  95
Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
        115                 120
```

<210> 3
<211> 28
<212> PRT
<213> Artificial Sequence
<220>
<223> The signal sequence from V. cholera classic strain 569B
<400> 3

```
Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
 1                5                  10                  15
Leu Cys Ala His Gly Ala Pro Gly Tyr Ala His Gly
            20                  25
```

<210> 4
<211> 124
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(124)
<223> CTB from variant NCBI gen bank number GI: 758351
<400> 4

```
Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser
 1                5                  10                  15
Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu
            20                  25                  30
Ser His Asn Thr Gln Ile Tyr Thr Leu Asn Asp Lys Ile Phe Ser Tyr
        35                  40                  45
Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys
    50                  55                  60
Asn Gly Ala Ile Phe Gln Val Glu Val Pro Ser Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala
                85                  90                  95
Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
        115                 120
```

<210> 5
<211> 103
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(103)
<223> CTB variant from NCBI gene bank GI: 1827850.
<400> 5

```
Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
 1                5                  10                  15
Ile Tyr Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala
            20                  25                  30
```

```
Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Ile Phe
        35                  40                  45
Gln Val Glu Val Pro Ser Ser Gln His Ile Asp Ser Gln Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala
65                  70                  75                  80
Lys Val Glu Lys Leu Cys Thr Trp Asn Asn Lys Thr Pro His Ala Ile
                85                  90                  95
Ala Ala Ile Ser Met Ala Asn
            100
```

<210> 6
<211> 124
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(124)
<223> CTB variant from NCBI gene bank GI: 808900.

```
<400> 6
Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser
 1               5                  10                  15
Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu
            20                  25                  30
Tyr His Asn Thr Gln Ile His Thr Leu Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Leu Ala Gly Asn Arg Glu Met Ala Ile Ile Thr Phe Lys
    50                  55                  60
Asn Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala
                85                  90                  95
Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
        115                 120
```

<210> 7
<211> 103
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(103)
<223> CTB variant from NCBI gene bank GI: 229616.

```
<400> 7
Thr Pro Glu Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
 1               5                  10                  15
Ile His Thr Leu Asn Asn Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala
            20                  25                  30
Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asp Gly Ala Thr Phe
        35                  40                  45
Glu Val Glu Val Pro Gly Ser Glu His Ile Asp Ser Glu Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala
65                  70                  75                  80
Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro His Ala Ile
                85                  90                  95
Ala Ala Ile Ser Met Ala Asn
            100
```

<210> 8
<211> 103
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(103)
<223> CTB variant from NCBI gene bank Gl: 998409.
<400> 8

```
Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
 1               5                  10                  15
Ile Tyr Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala
            20                  25                  30
Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Ile Phe
        35                  40                  45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala
65                  70                  75                  80
Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro His Ala Ile
                85                  90                  95
Ala Ala Ile Ser Met Ala Asn
            100
```

<210> 9
<211> 124
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(124)
<223> CTB variant from NCBI gene bank GI: 2144685.
<400> 9

```
Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser
 1               5                  10                  15
Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu
            20                  25                  30
Ser His Asn Thr Gln Ile Tyr Thr Leu Asn Asp Lys Ile Phe Ser Tyr
        35                  40                  45
Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys
    50                  55                  60
Asn Gly Ala Ile Phe Gln Val Glu Val Pro Ser Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala
                85                  90                  95
Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
        115                 120
```

<210> 10
<211> 103
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(103)
<223> CTB variant from NCBI gene bank GI: 1421511.
<400> 10

```
Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
 1               5                  10                  15
```

```
Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala
            20                  25                  30
Asp Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Thr Phe
        35                  40                  45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala
65                  70                  75                  80
Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro His Ala Ile
                85                  90                  95
Ala Ala Ile Ser Met Ala Asn
            100
```

<210> 11
<211> 124
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(124)
<223> CTB variant from NCBI gene bank GI: 48890(classic 569B).

```
<400> 11
Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser
 1               5                  10                  15
Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu
            20                  25                  30
Tyr His Asn Thr Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr
        35                  40                  45
Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys
    50                  55                  60
Asn Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala
                85                  90                  95
Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
        115                 120
```

<210> 12
<211> 105
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(105)
<223> CTB variant from NCBI gene bank GI: 2781121(Ogawa 41).

```
<400> 12
Met Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr
 1               5                  10                  15
Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu
            20                  25                  30
Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Thr
        35                  40                  45
Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys
    50                  55                  60
Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu
65                  70                  75                  80
Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro Ile Ile
```

85 90 95
Ala Ile Ala Ala Ile Ser Met Ala Asn
100 105

<210> 13
<211> 103
<212> PRT
<213> Vibrio cholera
<220>
<221> VARIANT
<222> (1)...(103)
<223> CTB variant from NCBI gene bank GI: 1421525(Ogawa 41 R35D).
<400> 13
Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
1 5 10 15
Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala
20 25 30
Gly Lys Asp Glu Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Thr Phe
35 40 45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
50 55 60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala
65 70 75 80
Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro His Ala Ile
85 90 95
Ala Ala Ile Ser Met Ala Asn
100

<210> 14
<211> 21
<212> PRT
<213> Artificial Sequence
<220>
<223> The signal sequence from LTB.
<400> 14
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
1 5 10 15
Leu Cys Ala Tyr Gly
20

<210> 15
<211> 125
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(125)
<223> LTB variant from NCBI gene bank GI:3062900.
<400> 15
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
1 5 10 15
Leu Ala Cys Ala Tyr Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser
20 25 30
Glu Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser
35 40 45
Tyr Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe
50 55 60
Lys Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile
65 70 75 80

```
Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile
                85                  90                  95
Thr Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn
            100                 105                 110
Lys Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
        115                 120                 125
```

<210> 16
<211> 124
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(124)
<223> LTB variant from NCBI gene bank GI:1169505.
<400> 16

```
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
 1               5                  10                  15
Leu Tyr Ala His Gly Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu
            20                  25                  30
Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
    50                  55                  60
Ser Gly Glu Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                85                  90                  95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Lys Asn
        115                 120
```

<210> 17
<211> 123
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(123)
<223> LTB variant from NCBI gene bank GI:1395122.
<400> 17

```
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
 1               5                  10                  15
Leu Cys Ala Tyr Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu
            20                  25                  30
Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
    50                  55                  60
Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                85                  90                  95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro Asn Ser Ile Ala Ala Ile Met Glu Asn
        115                 120
```

<210> 18
<211> 124
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(124)
<223> LTB variant from NCBI gene bank GI:145833.
<400> 18

```
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
 1               5                  10                  15
Leu Tyr Ala His Gly Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu
            20                  25                  30
Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
    50                  55                  60
Ser Gly Glu Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                85                  90                  95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Lys Asn
        115                 120
```

<210> 19
<211> 124
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(124)
<223> LTB variant from NCBI gene bank GI:1648865 (LT87).
<400> 19

```
Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
 1               5                  10                  15
Leu Cys Ala His Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu
            20                  25                  30
Tyr His Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
    50                  55                  60
Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                85                  90                  95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
        115                 120
```

<210> 20
<211> 123
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(123)
<223> LTB variant from NCBI gene bank GI:223254.

<400> 20
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
1 5 10 15
Leu Tyr Ala His Gly Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu
20 25 30
Tyr Arg Asn Thr Gln Ile Tyr Thr Asn Asp Lys Ile Leu Ser Tyr Thr
35 40 45
Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Met Ser
50 55 60
Gly Glu Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser
65 70 75 80
Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr Tyr
85 90 95
Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys Thr
100 105 110
Pro Asn Ser Ile Ala Ala Ile Ser Met Lys Asn
115 120

<210> 21
<211> 124
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(124)
<223> LTB variant from NCBI gene bank GI:408996.
<400> 21
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
1 5 10 15
Leu Cys Ala Tyr Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu
20 25 30
Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
35 40 45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
50 55 60
Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65 70 75 80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
85 90 95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
100 105 110
Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
115 120

<210> 22
<211> 103
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(103)
<223> LTB variant from NCBI gene bank GI:494265.
<400> 22
Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu Tyr Arg Asn Thr Gln
1 5 10 15
Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr Thr Glu Ser Met Ala
20 25 30
Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys Ser Gly Glu Thr Phe
35 40 45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
50 55 60

```
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr Tyr Leu Thr Glu Thr
65                  70                  75                  80
Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys Thr Pro Asn Ser Ile
                85                  90                  95
Ala Ala Ile Ser Met Lys Asn
            100
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<210> 23
<211> 124
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
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<223> LTB variant from NCBI gene bank GI:69630.
<400> 23

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 1               5                  10                  15
Leu Tyr Ala His Gly Ala Pro Gln Thr Ile Thr Gln Leu Cys Ser Glu
            20                  25                  30
Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Met
    50                  55                  60
Ser Gly Glu Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                85                  90                  95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Lys Asn
        115                 120
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<210> 24
<211> 123
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(123)
<223> LT-IIa from NCBI gene bank GI:146671.
<400> 24

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 1               5                  10                  15
Leu Ser Gly Gln Val Tyr Ala Gly Val Ser Glu His Phe Arg Asn Ile
            20                  25                  30
Cys Asn Gln Thr Thr Ala Asp Ile Val Ala Gly Val Gln Leu Lys Lys
        35                  40                  45
Tyr Ile Ala Asp Val Asn Thr Asn Thr Arg Gly Ile Tyr Val Val Ser
    50                  55                  60
Asn Thr Gly Gly Val Trp Tyr Ile Pro Gly Gly Arg Asp Tyr Pro Asp
65                  70                  75                  80
Asn Phe Leu Ser Gly Glu Ile Arg Lys Thr Ala Met Ala Ala Ile Leu
                85                  90                  95
Ser Asp Thr Lys Val Asn Leu Cys Ala Lys Thr Ser Ser Ser Pro Asn
            100                 105                 110
His Ile Trp Ala Met Glu Leu Asp Arg Glu Ser
        115                 120
```

<210> 25
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<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(122)
<223> LT-1Ib LTB from NCBI gene bank GI:576584.
<400> 25

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 1               5                  10                  15
Val Ser Val Gln Ala His Ala Gly Ala Ser Gln Phe Phe Lys Asp Asn
            20                  25                  30
Cys Asn Arg Thr Thr Ala Ser Leu Val Glu Gly Val Glu Leu Thr Lys
        35                  40                  45
Tyr Ile Ser Asp Ile Asn Asn Asn Thr Asp Gly Met Tyr Val Val Ser
    50                  55                  60
Ser Thr Gly Gly Val Trp Arg Ile Ser Arg Ala Lys Asp Tyr Pro Asp
65                  70                  75                  80
Asn Val Met Thr Ala Glu Met Arg Lys Ile Ala Met Ala Ala Val Leu
                85                  90                  95
Ser Gly Met Arg Val Asn Met Cys Ala Ser Pro Ala Ser Ser Pro Asn
            100                 105                 110
Val Ile Trp Ala Ile Glu Leu Glu Ala Glu
        115                 120
```

<210> 26
<211> 88
<212> PRT
<213> Shigella dysenteriae type 1
<220>
<221> VARIANT
<222> (1)...(88)
<223> Shiga toxin beta subunit from NCBI gene bank GI:152784.
<400> 26

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Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
 1               5                  10                  15
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
            20                  25                  30
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
        35                  40                  45
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
    50                  55                  60
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
65                  70                  75                  80
Gly Gly Phe Ser Val Ile Phe Arg
                85
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<210> 27
<211> 89
<212> PRT
<213> Escherichia coli
<220>
<221> VARIANT
<222> (1)...(89)
<223> Shiga-like toxin from E.coli from NCBI gene bank GI:4877349.
<400> 27

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 1               5                  10                  15
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Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
            20                  25                  30
Asn Glu Asn Asp Thr Phe Thr Val Lys Val Ala Gly Lys Glu Tyr Trp
        35                  40                  45
Thr Asn Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
    50                  55                  60
Gly Met Thr Val Thr Ile Lys Ser Asn Thr Cys Ala Ser Gly Ser Gly
65                  70                  75                  80
Phe Ala Glu Val Gln Phe Asn Asn Asp
                85
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<210> 28
<211> 226
<212> PRT
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<220>
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<222> (1)...(226)
<223> Pertussis Toxin pentamer subunit S2.
<400> 28

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 1                5                  10                  15
Leu Ala Leu Leu Gly Ser His Val Ala Arg Ala Ser Thr Pro Gly Ile
            20                  25                  30
Val Ile Pro Pro Gln Glu Gln Ile Thr Gln His Gly Ser Pro Tyr Gly
        35                  40                  45
Arg Cys Ala Asn Lys Thr Arg Ala Leu Thr Val Ala Glu Leu Arg Gly
    50                  55                  60
Ser Gly Asp Leu Gln Glu Tyr Leu Arg His Val Thr Arg Gly Trp Ser
65                  70                  75                  80
Ile Phe Ala Leu Tyr Asp Gly Thr Tyr Leu Gly Gly Glu Tyr Gly Gly
                85                  90                  95
Val Ile Lys Asp Gly Thr Pro Gly Gly Ala Phe Asp Leu Lys Thr Thr
            100                 105                 110
Phe Cys Ile Met Thr Thr Arg Asn Thr Gly Gln Pro Ala Thr Asp His
        115                 120                 125
Tyr Tyr Ser Asn Val Thr Ala Thr Arg Leu Leu Ser Ser Thr Asn Ser
    130                 135                 140
Arg Leu Cys Ala Val Phe Val Arg Ser Gly Gln Pro Val Ile Gly Ala
145                 150                 155                 160
Cys Thr Ser Pro Tyr Asp Gly Lys Tyr Trp Ser Met Tyr Ser Arg Leu
                165                 170                 175
Arg Lys Met Leu Tyr Leu Ile Tyr Val Ala Gly Ile Ser Val Arg Val
            180                 185                 190
His Val Ser Lys Glu Glu Gln Tyr Tyr Asp Tyr Glu Asp Ala Thr Phe
        195                 200                 205
Glu Thr Tyr Ala Leu Thr Gly Ile Ser Ile Cys Asn Pro Gly Ser Ser
    210                 215                 220
Leu Cys
225
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<210> 29
<211> 227
<212> PRT
<213> Bordetella pertussis
<220>
<221> VARIANT
<222> (1)...(227)
<223> Pertussis Toxin pentamer subunit S3.

<400> 29
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1 5 10 15
Val Leu Ala Leu Leu Gly Met Arg Thr Ala Gln Ala Val Ala Pro Gly
20 25 30
Ile Val Ile Pro Pro Lys Ala Leu Phe Thr Gln Gln Gly Gly Ala Tyr
35 40 45
Gly Arg Cys Pro Asn Gly Thr Arg Ala Leu Thr Val Ala Glu Leu Arg
50 55 60
Gly Asn Ala Glu Leu Gln Thr Tyr Leu Arg Gln Ile Thr Pro Gly Trp
65 70 75 80
Ser Ile Tyr Gly Leu Tyr Asp Gly Thr Tyr Leu Gly Gln Ala Tyr Gly
85 90 95
Gly Ile Ile Lys Asp Ala Pro Pro Gly Ala Gly Phe Ile Tyr Arg Glu
100 105 110
Thr Phe Cys Ile Thr Thr Ile Tyr Lys Thr Gly Gln Pro Ala Ala Asp
115 120 125
His Tyr Tyr Ser Lys Val Thr Ala Thr Arg Leu Leu Ala Ser Thr Asn
130 135 140
Ser Arg Leu Cys Ala Val Phe Val Arg Asp Gly Gln Ser Val Ile Gly
145 150 155 160
Ala Cys Ala Ser Pro Tyr Glu Gly Arg Tyr Arg Asp Met Tyr Asp Ala
165 170 175
Leu Arg Arg Leu Leu Tyr Met Ile Tyr Met Ser Gly Leu Ala Val Arg
180 185 190
Val His Val Ser Lys Glu Glu Gln Tyr Tyr Asp Tyr Glu Asp Ala Thr
195 200 205
Phe Gln Thr Tyr Ala Leu Thr Gly Ile Ser Leu Cys Asn Pro Ala Ala
210 215 220
Ser Ile Cys
225

<210> 30
<211> 152
<212> PRT
<213> Bordetella pertussis
<220>
<221> VARIANT
<222> (1)...(152)
<223> Pertussis Toxin pentamer subunit S4.
<400> 30
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1 5 10 15
Ala Arg Arg Ser Arg Val Arg Ala Leu Ala Trp Leu Leu Ala Ser Gly
20 25 30
Ala Met Thr His Leu Ser Pro Ala Leu Ala Asp Val Pro Tyr Val Leu
35 40 45
Val Lys Thr Asn Met Val Val Thr Ser Val Ala Met Lys Pro Tyr Glu
50 55 60
Val Thr Pro Thr Arg Met Leu Val Cys Gly Ile Ala Ala Lys Leu Gly
65 70 75 80
Ala Ala Ala Ser Ser Pro Asp Ala His Val Pro Phe Cys Phe Gly Lys
85 90 95
Asp Leu Lys Arg Pro Gly Ser Ser Pro Met Glu Val Met Leu Arg Ala
100 105 110
Val Phe Met Gln Gln Arg Pro Leu Arg Met Phe Leu Gly Pro Lys Gln
115 120 125
Leu Thr Phe Glu Gly Lys Pro Ala Leu Glu Leu Ile Arg Met Val Glu
130 135 140
Cys Ser Gly Lys Gln Asp Cys Pro
145 150

<210> 31
<211> 133
<212> PRT
<213> Bordetella pertussis
<220>
<221> VARIANT
<222> (1)...(133)
<223> Pertussis Toxin pentamer subunit S5.
<400> 31

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Met Gln Arg Gln Ala Gly Leu Pro Leu Lys Ala Asn Pro Met His Thr
 1               5                  10                  15
Ile Ala Ser Ile Leu Leu Ser Val Leu Gly Ile Tyr Ser Pro Ala Asp
            20                  25                  30
Val Ala Gly Leu Pro Thr His Leu Tyr Lys Asn Phe Thr Val Gln Glu
        35                  40                  45
Leu Ala Leu Lys Leu Lys Gly Lys Asn Gln Glu Phe Cys Leu Thr Ala
    50                  55                  60
Phe Met Ser Gly Arg Ser Leu Val Arg Ala Cys Leu Ser Asp Ala Gly
65                  70                  75                  80
His Glu His Asp Thr Trp Phe Asp Thr Met Leu Gly Phe Ala Ile Ser
                85                  90                  95
Ala Tyr Ala Leu Lys Ser Arg Ile Ala Leu Thr Val Glu Asp Ser Pro
            100                 105                 110
Tyr Pro Gly Thr Pro Gly Asp Leu Leu Glu Leu Gln Ile Cys Pro Leu
        115                 120                 125
Asn Gly Tyr Cys Glu
    130
```

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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PCT

(10) International Publication Number
WO 01/27144 A3

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(75) Inventors/Applicants *(for US only)*: **HANDLEY, Harold, H.** [US/US]; 705 Arden Drive, Encinitas, CA 92024 (US). **HAAPARANTA, Tapio** [SE/US]; 7777 Forrestal Road, San Diego, CA 92120 (US). **EWALT, Karla, L.** [US/US]; #144, 5275 Toscana Way, San Diego, CA 92122 (US).

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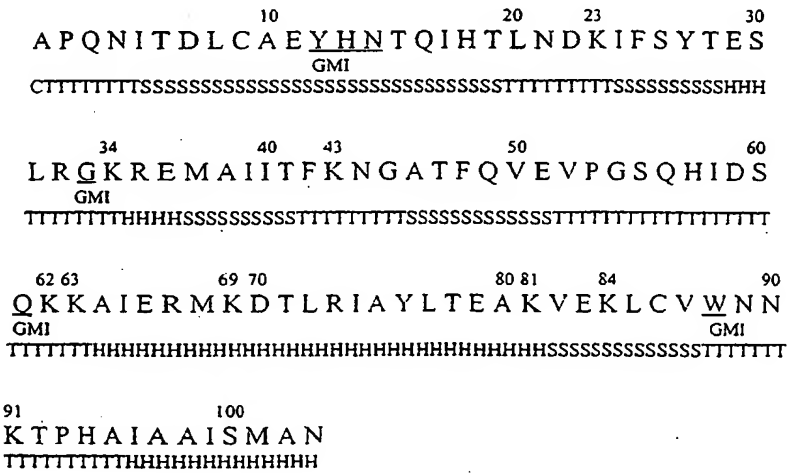
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Continued on next page]

(54) Title: AB_5 TOXIN B SUBUNIT MUTANTS WITH ALTERED CHEMICAL CONJUGATION CHARACTERISTICS

Amino acid sequence (SEQ ID NO: 2), predicted secondary structure, position of lysine residues and regions involved in binding to GMI



H=Helix; S=Sheet; T=Turn; C=Coil

- 9 lysine residues.
- 4 are at the top of CTB (23, 43, 81, 840).
- 4 are at the bottom of CTB, proximal to or in the GM1 binding site (34, 62, 63, 91).
- K69 is within the alpha helix.

(57) Abstract: A recombinant AB_5 B subunit protein including at least one mutation, wherein the mutation alters the number of amino acid residues available for chemical modification as compared to a wild type AB_5 B subunit protein, and wherein said recombinant protein retains an effective target ligand bind affinity. For example, specifically designed mutations are produced in the cholera Toxin B subunit (CTB) such that it can still bind with high affinity to its receptor, Gm-1, but can be specifically covalently linked at lysines or cysteines to an immunogen or vaccine. The vaccine produced from this coupling is a mucosal vaccine which has high immunogenicity due to the interaction with the CTB. The vaccine can be produced inexpensively and easily. Alternatively, a technique is disclosed for treating CTB such that non-covalent coupling to a vaccine or immunogen can occur.

WO 01/27144 A3

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) **Date of publication of the international search report:**
17 January 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 00/27607

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/28 C07K14/245 C07K14/25 C07K14/235 C12N15/31
C12N5/10 A61K39/106 A61K39/112 A61K39/10 A61K39/108

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOBLING M G ET AL: "ANALYSIS OF STRUCTURE AND FUNCTION OF THE B SUBUNIT OF CHOLERA TOXIN BY THE USE OF SITE-DIRECTED MUTAGENESIS" MOLECULAR MICROBIOLOGY, vol. 55, no. 7, 1991, pages 1755-1767, XP000992490 ISSN: 0950-382X	1,3-5, 7-13,15, 24-33,42
Y	page 1761, right-hand column, paragraph 2 -left-hand column, paragraph 1; table 2 ---	27-41, 43,44
Y	US 5 834 246 A (SANCHES CASTILLO JOAQUIN ET AL) 10 November 1998 (1998-11-10) examples 1-3 ---	27-33
Y	EP 0 556 388 A (TORAY INDUSTRIES) 25 August 1993 (1993-08-25) page 3 -page 4 ---	34-41
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2001	28. 06. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ALCONADA RODRIG.., A

4

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BACKSTROM M ET AL: "Characterization of an internal permissive site in the cholera toxin B-subunit and insertion of epitopes from human immunodeficiency virus-1, hepatitis B virus and enterotoxigenic Escherichia coli" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 165, no. 2, 20 November 1995 (1995-11-20), pages 163-171, XP004043137 ISSN: 0378-1119 the whole document	43,44
Y	FR 2 636 842 A (LIEGE UNIVERSITE ETAT) 30 March 1990 (1990-03-30) page 34, line 26 -page 40, line 15	43,44
A	DEBINSKI W ET AL: "AN IMMUNOTOXIN WITH INCREASED ACTIVITY AND HOMOGENEITY PRODUCED BY REDUCING THE NUMBER OF LYSINE RESIDUES IN RECOMBINANT PSEUDOMONASEXOTOXIN" BIOCONJUGATE CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY, WASHINGTON, vol. 5, no. 1, 1994, pages 40-46, XP000430384 ISSN: 1043-1802 the whole document	24,25
A	DE WOLF MARC J S ET AL: "Regeneration of active receptor recognition domains on the B subunit of cholera toxin by formation of hybrids from chemically inactivated derivatives." BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1223, no. 2, 1994, pages 285-295, XP000992489 ISSN: 0006-3002 figure 5; table 2	
A	MERRITT ETHAN A ET AL: "Crystal structure of cholera toxin B-pentamer bound to receptor G-M1 pentasaccharide." PROTEIN SCIENCE, vol. 3, no. 2, 1994, pages 166-175, XP000992494 ISSN: 0961-8368 figure 2; table 2	

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/US 00/27607

Box I Obs rvations where certain claims wer found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 34-41 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 20-23 (completely) and 1-13, 18 and 24-44 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,3-13,24-44(partially),14-17(complete)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 20-23 (completely) and 1-13, 18 and 24-44 (partially)

Present claims 1-13, 18 and 20-44 relate to recombinant variants of the cholera toxin B subunit polypeptide defined by reference to a desirable characteristic or property, namely, that they comprise at least one mutation, wherein said mutation alters the number of residues available for chemical modification as compared to wild-type B subunit protein, and wherein said recombinant proteins retains an effective target ligand binding afinity. The claims cover all polypeptide variants having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such variants. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the polypeptide variants by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the variants in which the number of lysines has been reduced by replacing lysines at position 62, 63 and/or 91 (see example 5 and claims 14-17) and relating to variants in which the number of amino acids susceptible of chemical modification (lysine or cysteine) has been increased by introducing the A1K, P2K, H18K, M101C and N103C mutations (example 3 and claim 19).

Present claims 1-7, 20, 21, 23-28, 30, 31, 33-36, 38-40, 42 and 43 relate to recombinant variants of the E.coli heat labile toxin B protein (LTB); the LT type IIa B protein, the LT type IIb protein, the Shiga toxin B protein, the Shiga-like toxin B protein and the pertussis toxin B protein defined by reference to a desirable characteristic or property, namely, that they comprise at least one mutation, wherein said mutation alters the number of residues available for chemical modification as compared to wild-type B subunit protein, and wherein said recombinant proteins retains an effective target ligand binding afinity. The claims cover all polypeptide variants having this characteristic or property, whereas the application provides neither support within the meaning of Article 6 PCT nor disclosure within the meaning of Article 5 PCT for any of such variants. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the polypeptide variants by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for those claims.

Present claims 20-23 and 34-41 relate to recombinant variants of the AB5 B subunits defined by reference to a desirable characteristic or

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

property, namely, that they comprise at least one mutation, that increases the number of residues available for chemical modification as compared to wild-type B subunit protein, and at least one mutation that decreases the number of residues available for chemical modification as compared to wild-type B subunit protein. The claims cover all polypeptide variants having this characteristic or property, whereas the application provides neither support within the meaning of Article 6 PCT nor disclosure within the meaning of Article 5 PCT for any of such variants. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the polypeptide variants by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for those claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1,3-13,24-44 (partially) and 14-17 (complete)

A recombinant cholera toxin B subunit protein comprising at least one mutation that leads to a decreased number of residues susceptible of chemical modification in the vicinity of a GMI binding-site; the gene encoding said B subunit; a method of making said recombinant B subunit gene; a method of producing the recombinant B subunit protein; a method of generating an immune response to said recombinant B subunit; expression vectors comprising the gene encoding the recombinant B subunit and the gene encoding for the B subunit fused to a immunogenic peptide.

2. Claims: 1,2,4-12,18,24-44 (partially) and 19 (complete)

A recombinant cholera toxin B subunit protein comprising at least one mutation that leads to an increased number of residues susceptible of chemical modification; the gene encoding said B subunit; a method of making said recombinant B subunit gene; a method of producing the recombinant B subunit protein; a method of generating an immune response to said recombinant B subunit; expression vectors comprising the gene encoding the recombinant B subunit and the gene encoding for the B subunit fused to a immunogenic peptide.

INTERNATIONAL SEARCH REPORT

formation on patent family members

International Application No
PC1,/JS 00/27607

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5834246 A	10-11-1998	US 5268276 A	07-12-1993
		US 6043057 A	28-03-2000
EP 0556388 A	25-08-1993	JP 2115129 A	27-04-1990
		WO 9004405 A	03-05-1990
FR 2636842 A	30-03-1990	AT 123525 T	15-06-1995
		DE 68923006 D	13-07-1995
		EP 0445128 A	11-09-1991
		WO 9003437 A	05-04-1990